UNIVERSITY OF ALBERTA

APPLICATION OF MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY TO THE STUDY OF BACTERIOCINS

by

Natisha Linda Rose

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Application of Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry to the Study of Bacteriocins** submitted by Natisha Linda Rose in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Science and Technology.

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ABSTRACT

Bacteriocins produced by lactic acid bacteria have the potential for use as natural preservatives in minimally processed foods. However, sensitive detection methods are needed to understand their role in the enhanced safety and/or storage life of foods and to clarify their fate in foods. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was examined as a method of detection of bacteriocins from crude biological samples. After minimal sample clean-up to remove natural contaminants that interfere with MS analysis, nisin A, pediocin AcH/PA-1, brochocins A and B and enterocins A and B were detected in culture supernatants using MALDI-TOF MS. This technology was then applied to determine the fate of nisin in fresh meat. Nisin is widely used in the food industry; however, it is ineffective for use in raw meat products. Fresh and cooked meat samples were held in a nisin solution for 1 hr, vacuum packaged and stored overnight at 4°C. The extracts of the meat were analyzed by antibacterial assays and by MALDI-TOF MS. Antibacterial activity was recovered from the cooked meat extracts; whereas no activity was recovered from the raw meat extracts. MALDI-TOF MS results for the cooked meat extract showed a signal at the molecular weight of nisin; whereas only a signal that was 307 Da greater than the mass of nisin was observed for the raw meat extracts. These results suggested that nisin was inactivated in raw meat by an enzymatic reaction with glutathione, a low molecular weight (307 Da) thiol compound that is widely distributed in plant and animal tissues. To further examine this theory, soluble glutathione S-transferase was isolated from fresh beef by affinity chromatography. The *in vitro* reaction between nisin and glutathione, catalyzed by the

purified enzyme, was then studied. The products of the reaction were analyzed for antibacterial activity and by MALDI-TOF MS. Results showed that after reaction with glutathione, nisin lost its antibacterial activity. MALDI-TOF MS results showed that the reaction could result in the addition of up to three glutathione molecules to one nisin molecule. Glutathione assays with nisin variants and fragments confirmed that the dehydroalanine residues of nisin were two sites of addition for glutathione. It is speculated that dehydrobutyrine is the third site of addition of glutathione to nisin. These results provided the first scientific evidence for the method of inactivation of nisin in fresh meat.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
APT	All-purpose Tween
ATP	Adenosine tri-phosphate
AU	Arbitrary units
BSA	Bovine serum albumin
C	Celsius
CDNB	1-chloro-2,4-nitrobenzene
CO_2	Carbon dioxide
Da	Dalton(s)
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DNA	Deoxyribonucleic acid
GSH	Glutathione
GST	Glutathione S-transferase
h	Hour(s)
HCCA	α-cyano-4-hydroxycinnamic acid
HCl	Hydrochloric acid
HM	High mass
HPLC	High performance liquid chromatography
IU	International unit
KE	Kinetic energy

L	Liter
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography-mass spectrometry
LM	Low mass
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
min	Minute(s)
mL	Milliliter
MRS	de Man, Rogosa and Sharpe
MW	Molecular weight
m/z	Mass/charge
N_2	Nitrogen
nm	Nanometer(s)
NMR	Nuclear magnetic Resonance
PAGE	Polyacrylamide gel electrophoresis
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SH	Sulfhydryl group
TFA	Trifluoroacetic acid
TPYG	Trypticase peptone yeast glucose
v/v	volume/volume
w/v	weight/volume

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction.

Current trends in the food industry are focused on using packaging technology and natural preservatives to increase the storage life and enhance the safety of foods. One such advance in packaging technology has been the introduction of modified atmosphere packaging, which involves packaging products in varying concentrations of carbon dioxide, nitrogen and oxygen. The extended storage life provided by modified atmosphere packaging is partially due to the presence of a lactic acid bacteria (LAB) microflora. LAB provide an adventitious environment through the production of a number of inhibitory compounds, including organic acids and antibacterial peptides called bacteriocins. Although the storage life of such packaged foods can be extended to several months, there is a concern that foodborne pathogens, such as *Listeria monocytogenes*, can thrive in such an environment.

Controlling the microflora that develops on modified atmosphere packaged foods could be achieved by natural means with bacteriocin-producing LAB. To achieve the successful application of bacteriocins, clarification of the fate of bacteriocins in food products is necessary to understand their role in the enhanced safety or storage life. However, such understanding is not possible without the ability to detect bacteriocins in foods at very low concentrations. This literature review will provide an overview of the potential for the use of LAB and bacteriocins in foods, the successful application of the

bacteriocin nisin to foods, and a potential method for a sensitive and rapid method of detection of bacteriocins.

1.2. The lactic acid bacteria.

Lactic acid bacteria are amongst a group of Gram-positive bacteria that have enjoyed successful use in the food industry for hundreds of years. Numerous studies on LAB have resulted in an extensive knowledge base of their characteristics and applications. Traditionally, bacterial taxonomy was determined by morphological and physiological characteristics. LAB were characterized as Gram-positive, non-motile, non-spore forming, rod- and coccus-shaped organisms that ferment carbohydrates and alcohols, with the primary fermentation end product being lactic acid (Orla-Jensen, 1999). Current approaches on LAB classification include cell characteristics (e.g. cell wall composition and cellular fatty acids) and molecular characteristics (e.g. mol% G+C content of DNA and ribosomal RNA sequencing; Stiles and Holzapfel, 1997). Presently, LAB are defined as gram-positive anaerobic bacteria that are catalase-negative, non-spore forming, cocci, coccobacilli or rods that have <55 mol% G+C content in their DNA (Stiles and Holzapfel, 1997).

LAB have been isolated as indigenous bacteria from a variety of minimally processed foods (Garver and Muriana, 1993). This, and the common addition to food, including dairy and meat products, as starter cultures have resulted in GRAS (generally regarded as safe) status of LAB. The primary means of preservation by LAB is through fermentative acidification. End products of LAB fermentation include the accumulation of organic acids, primarily lactic and acetic acids, which result in a reduction of pH

(Lindgren and Dobrogosz, 1990). Other fermentation end products that can aid in the inhibition of microorganisms are ethanol, carbon dioxide, hydrogen peroxide, formic acid, acetoin, 2,3-butanediol, and diacetyl. Several LAB have also been shown to produce antibacterial peptides called bacteriocins.

1.3. Bacteriocins produced by lactic acid bacteria.

Bacteriocins are defined as peptides or proteins produced by bacteria that exhibit antibacterial activity against closely related strains (Klaenhammer, 1993). The classification system most commonly used for bacteriocins is based on the initial definition of a proteinaceous inhibitor, an estimation of the molecular mass, and the determination of susceptible strains (Klaenhammer, 1993). On this basis, 4 classes of bacteriocins have been defined: class I, lantibiotics which are small membrane-active peptides (<5 kDa) containing unusual amino acids such as lanthionine, β-methyllanthionine and dehydrated residues; class II, small heat-stable non-lanthionine containing membrane-active proteins (<10 kDa) characterized by a double glycine processing site in the bacteriocin precursor peptide; class III, large, heat-labile proteins (>30 kDa), and class IV, complex bacteriocins composed of protein with one or more lipid and/or carbohydrate moieties required for activity. Class II bacteriocins are further subdivided into 3 subclasses: class IIa, *Listeria*-active peptides with a consensus sequence in the N-terminal of YGNGV; class IIb, two-peptide bacteriocins, and class IIc, thiol-activated peptides requiring cysteine residues for activity. Nes et al. (1996) used a similar classification method with the exceptions of describing class IIa bacteriocins as pediocin-like bacteriocins with strong antilisterial activity and class IIc as sec-dependent secreted

bacteriocin. Others have proposed alternative classifications for bacteriocins (van Belkum and Stiles, 2000). Amongst the different classes of bacteriocins, the lantibiotics are the most extensively studied because of the successful application of the lantibiotic nisin in the food industry.

1.4. The lantibiotics.

Lantibiotics, so-called because of their characteristic thio-ether bridges, are subdivided into 2 groups based on their primary spatial structures and mode of action (Engelke et al., 1992; Sahl, 1994). Type A lantibiotics are elongated and exhibit an overall net positive charge. The characteristic shape is the result of ring formation from a C-terminal cysteine residue to an N-terminal dehydrated amino acid (Sahl, 1994). Killing action of type A lantibiotics is typically by means of pore formation that induces the leakage of ions and ultimately results in the loss of the electrochemical proton gradient. Type B lantibiotics have a characteristic globular shape that is the result of head-to-tail bridging with lanthionines that can be formed from either a C-terminal or N-terminal cysteine. These lantibiotics have a net charge of –1 or 0. The antibacterial activity of type B lantibiotics is by inhibition of specific enzymes. A list of well-characterized type A and type B lantibiotics is listed in Table 1.1. Structures showing the typical linear shape of type A lantibiotics and globular shape of type B lantibiotics are in Figure 1.1.

1.5. Nisin.

Among the lantibiotics, nisin is the most extensively studied because it is the first bacteriocin to have achieved commercial application in the food industry. The ability of

nisin to withstand heat and acid make it attractive for use in foods. However, its potential is also limited by its instability at higher pH. To date, nisin is licensed for use as a food additive in dairy products, canned foods and vegetables in over 50 countries (Delves-Broughton et al., 1996).

Table 1.1. Examples of type A and type B lantiobiotics. Adapted from Sahl and Bierbaum (1998).

Lantibiotic	Producing species	Molecular mass (Da)	% Modified residues	# of rings
Type A Lantiobiotics				
Nisin A	*L. lactis* subsp. *lactis*	3353	38	5
Nisin Z	*L. lactis* subsp. *lactis*	3330	38	5
Subtilin	*Bacillus subtilis*	3317	40	5
Epidermin	*Staphylococcus epidermidis*	2164	41	4
Gallidermin	*Staphylococcus gallinarum*	2164	41	4
[Val1, Leu6]-epidermin	*Staphylococcus epidermidis*	2151	41	4
Mutacin B-Ny266	*Streptococcus mutans*	2270	41	4
Pep5	*Staphylococcus epidermidis*	3488	26	3
Epidicin 280	*Staphylococcus epidermidis*	3133	27	3
Epilancin K7	*Staphylococcus epidermidis*	3032	32	3
Lactocin S	*Lactobacillus sakei*	3764	24	2
SA-FF22	*Streptococcus pyogenes*	2795	27	3
Lactacin 481	*L. lactis*	2901	26	3
Salivaricin A	*Streptococcus salivarius*	2315	27	3
[Lys2,Phe7]-Salivaricin A	*Streptococcus salivarius*	2321	27	3
Variacin	*Micrococcus varians*	2658	28	3
Cypemycin	*Streptomyces* spp.	2094	41	1
Type B Lantibiotics				
Cinnamycin	*Streptomyces cinnamoneus*	2042	47	4
Duramycin	*Streptomyces cinnamoneus*	2014	47	4
Duramycin B	*Streptoverticillium* spp.	1951	47	4
Duramycin C	*Streptomyces griseoluteus*	2008	47	4
Ancovenin	*Streptomyces* spp.	1959	37	3
Mersacidin	*Bacillus* spp.	1825	42	4
Actagardine	*Actinoplanes* spp.	1890	45	4

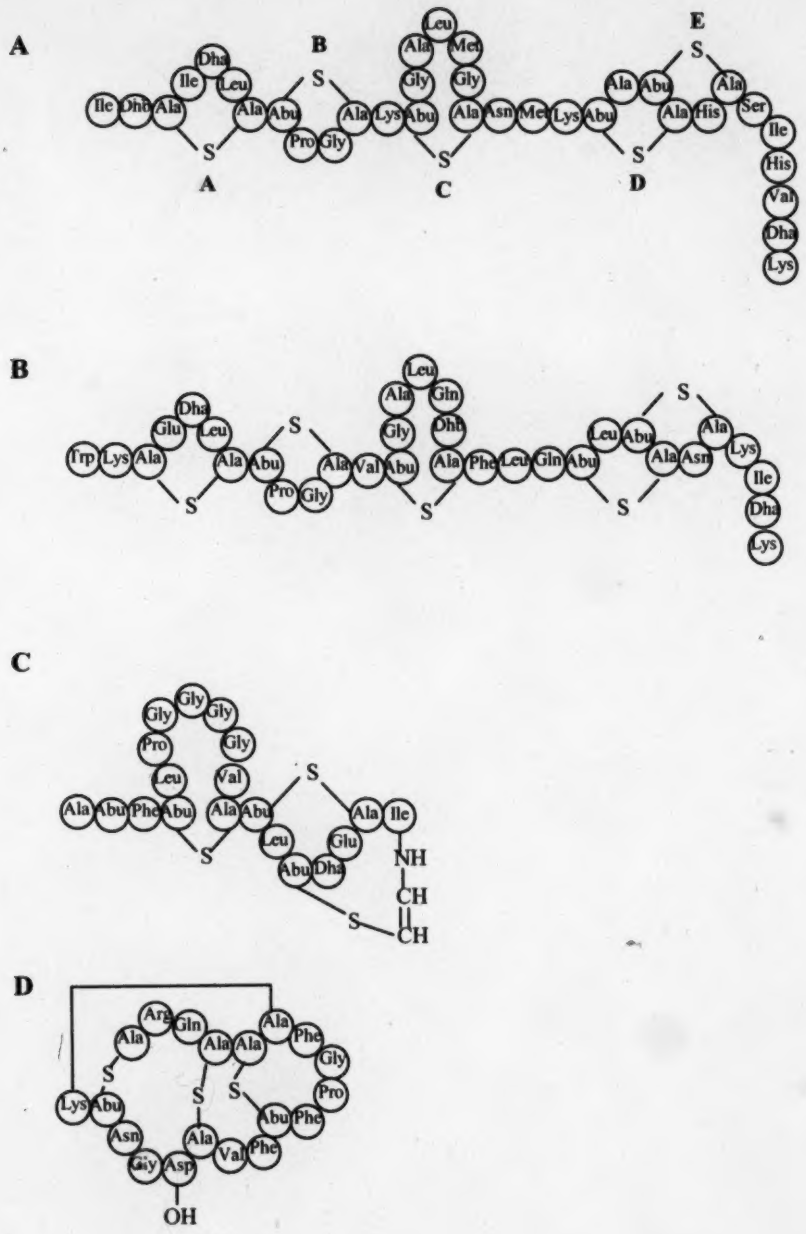

Figure 1.1. Structural examples of type A lantibiotics nisin A, defining rings A-E (A) and subtilin (B) and type B lantibiotics mersacidin (C) and cinnamycin (D).

Nisin activity was first reported in 1928 (Rogers, 1928). It was later described as a protein or polypeptide after Whitehead (1933) isolated it from a fermentation of a streptococcal strain that was inhibitory to starter cultures used in cheese production. Nisin producers were among the lactic streptococci belonging to serological group N. The term nisin was coined in 1947 by Mattick and Hirsch (1947) from the letters N Inhibitory Substance. In the 1980s, most of the serological group N lactic streptococci stains were reclassified to the genus *Lactococcus*.

1.5.1. *The biosynthesis of nisin.*

Biosynthesis of nisin requires an eleven-gene cluster, *nis*ABTCIPRKFEG (as shown in Figure 1.2) that is located on a 70 kb conjugative transposon. Ra et al. (1996) showed that this gene cluster is actually transcribed as two operons (*nis*ABTCIPRK and *nis*FEG) and each operon has its own promoter. They also demonstrated that both operons are induced by external nisin, and therefore, they form one regulon. In addition to the genes for nisin biosynthesis, the transposon also contains the genetic information for sucrose fermentation.

Figure 1.2. The gene cluster of *Lactococcus lactis* subsp. *lactis*. Adapted from Kleerebezem et al. (1999).

There are several discrepancies in the literature as to whether the nisin gene cluster is located on a plasmid or on the chromosome. Several plasmids of different sizes are known in *Lactococcus lactis* subsp. *lactis (L. lactis)*, and early studies suspected that nisin biosynthesis was plasmid-mediated (Hurst, 1981). Steen et al. (1991) looked to further characterize the locus of the genes involved in nisin biosynthesis and found them to be located on the chromosome. Kim et al. (1997) also noted that nisin production is encoded by a conjugative transposon in the host chromosome. However, they also reported that this transposon can be excised from the chromosome and stabilized as a plasmid. All transformed plasmids were nisin-producing, nisin-resistant and utilized sucrose.

Two natural variants of nisin produced by *L. lactis* have been identified. Nisin Z was compared with nisin A by de Vos et al. (1993). The first gene of the this gene cluster (referred to as *nis*Z in the case of nisin Z) is the structural gene. The structural genes of nisin A and nisin Z differ by one nucleotide substitution that results in an asparagine at position 27 instead of a histidine as in nisin A. The genes downstream from the structural gene have been shown to be involved in the posttranslational modification of nisin, proteolytic cleavage of the leader peptide, regulation of nisin biosynthesis, transport from the cell, and host strain immunity.

1.5.1.1. *The maturation of the nisin molecule*. The nisin A structural gene, *nis*A, was first identified as being plasmid-located and encoding for a 57 amino acid prepeptide (Kaletta and Entian, 1989). It was then suggested that this peptide was posttranslationally processed to form the unusual amino acid residues and cleaved at a characteristic site

(Arg –1, Ile +1). Two potential chemical reactions were proposed for the maturation of lantibiotics: 1) dehydration of serine and threonine residues and 2) addition of sulfur to form lanthionine bridges (Engelke et al., 1992). Figure 1.3 outlines the formation of a mature nisin molecule.

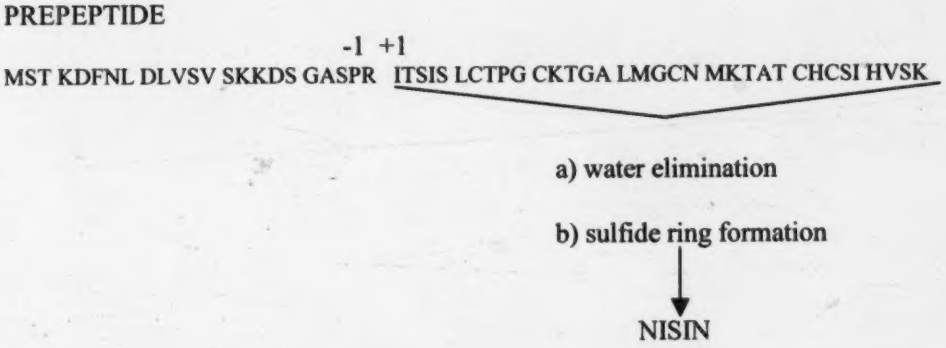

Figure 1.3. Outline of the maturation of the lantibiotic nisin as proposed by Kaletta and Entian (1989) and Engelke et al. (1992).

The posttranslational modifications discussed above involve the dehydration of either a serine or a threonine residue to produce a dehydroalanine or dehydrobutyrine residue, respectively. These residues can then react with neighboring cysteine residues to form thio-ether bonds resulting in lanthionine or β-methyllanthione residues, respectively. The chemical reactions forming these residues are shown in Figure 1.4.

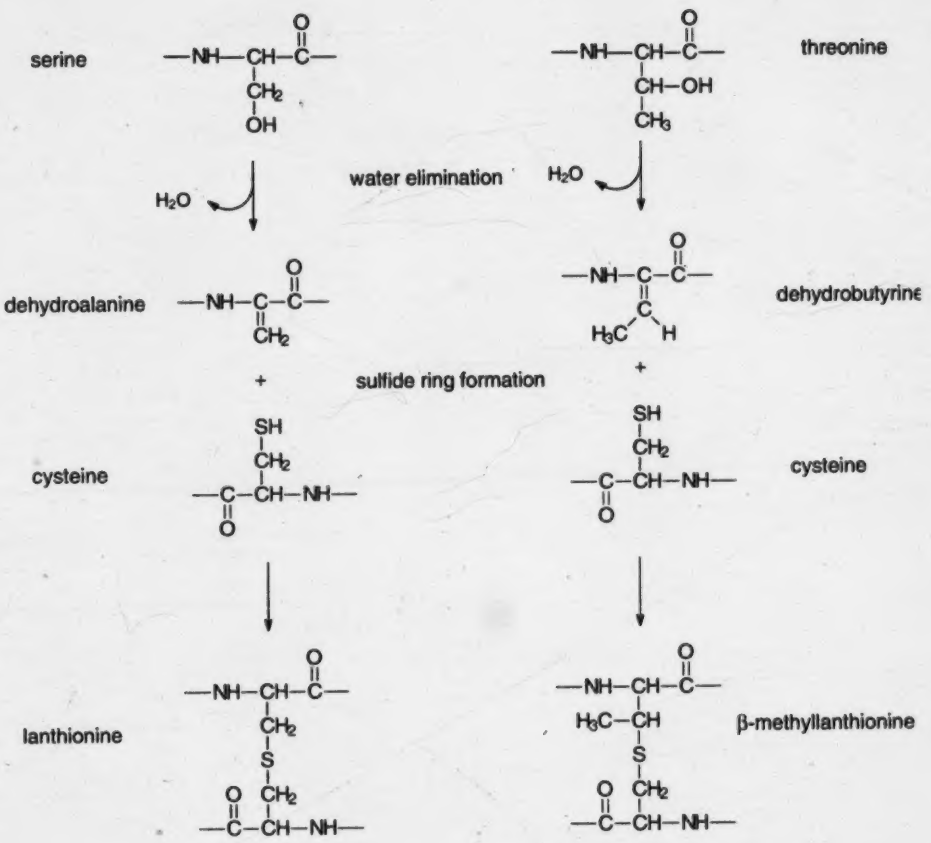

Figure 1.4. The chemical reactions involved in the posttranslational modification of the nisin molecule.

Several functions of the leader peptide have been proposed, including: 1) acting as a recognition site for biosynthetic enzymes; 2) preventing non-specific modifications of the mature nisin part; 3) contributing to host immunity by rendering the nisin molecule inactive, and 4) assisting in transporting the precursor peptide across the cell membrane (van der Meer et al., 1994; Jack and Sahl, 1995). The spatial structure of the leader peptide was studied to determine whether there were any reactions between the leader peptide and the lantibiotic portion of the precursor peptide. In aqueous solution, it was found that the leader peptide maintains a random coil structure and has no interaction with the nisin part of the precursor (van den Hooven et al., 1997). It is also known that no modifications occur in the leader part of the precursor peptide and the prenisin molecule does not exhibit antibacterial activity (van der Meer et al., 1993). Site-directed mutagenesis confirmed that cleavage of the precursor peptide is the final step in nisin maturation and that this cleavage is essential in rendering nisin active (van der Meer et al., 1994).

1.5.1.2. *General model for the biosynthesis of nisin.* Advances in the study of the nisin gene cluster have resulted in the proposal of several models for nisin biosynthesis (Sahl, 1994; Kuipers et al., 1995; Jack and Sahl, 1995; Entian and deVos, 1996; Siegers et al., 1996; Sahl and Bierbaum, 1998; Kleerebezem et al., 1999). A general model depicting the involvement of several proteins in nisin biosynthesis is shown in Figure 1.5.

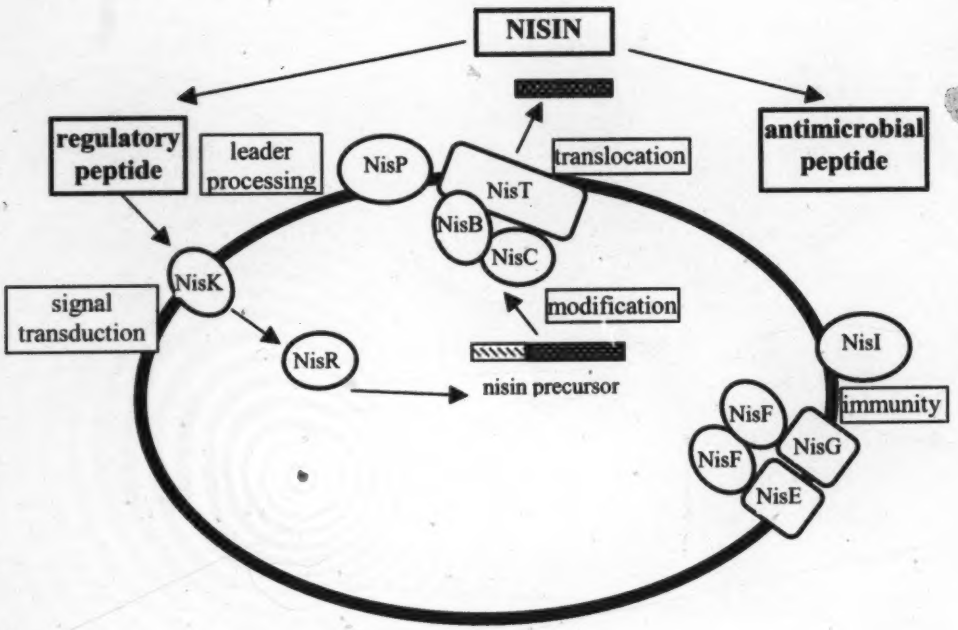

Figure 1.5. A general model for the biosynthesis of nisin. Adapted from Kleerebezem et al. (1999).

Adjacent to the *nis*A gene are the genes *nis*B, *nis*T and *nis*C. Early studies showed that the NisB protein is membrane-associated, and suggested that nisin biosynthesis is likely to occur at the cell membrane (Engelke et al., 1992). It was also suggested that the NisB and NisC proteins were involved in the posttranslational modification of nisin. In a sample of pure nisin, there is typically 10% of nisin that has not undergone dehydration at position 33 (Sen et al., 1999). Overexpression of the NisB protein resulted in greater efficiency of the dehydration of Ser33 to Dha33 and essentially, a form of fully processed

nisin molecule (Sen et al., 1999). Therefore, it is assumed that the NisB protein is involved in the dehydration of the serine and threonine molecules, and that perhaps the NisC protein is involved in the formation of the lanthionine and β-methyllanthionine residues. NisT showed homology to known transport proteins that are part of the ABC family, and therefore, it was assumed that it played a role in the transport of the nisin molecule across the cytoplasmic membrane (Engelke et al., 1992). Evidence of the involvement of NisT in transporting the nisin molecule out of the cell was provided by Qiao and Saris (1996). It was demonstrated that when the NisT protein was not expressed, nisin could not be detected in the culture supernatant; however, it could be detected in cell lysates. Siegers et al. (1996) termed the membrane-bound complex consisting of NisB, NisC and NisT as lanthionine synthase because of its role in the catalysis of the dehydration reactions, forming thio-ether bonds between the neighboring cysteines, and transporting the protein.

Engelke et al. (1994) reported the identification of 4 additional open reading frames that were adjacent and downstream from the *nis*B, *nis*T, and *nis*C encoding genes. The genes were: 1) *nis*I encoding a protein for immunity; 2) *nis*P encoding a protein to cleave the leader peptide, and 3) *nis*R and *nis*K encoding regulatory proteins. After cleavage of the precursor peptide by NisP, which renders a fully active nisin molecule, it is important for the cell to display immunity against that molecule for self-protection. The expression of the *nis*I gene in a nisin-sensitive *L. lactis* strain resulted in an active immune protein at levels comparable to a wild-type nisin-producing strain (Qiao et al., 1995). However, the level of immunity was only increased slightly, which suggests that other proteins were required for full immunity. Disruptions in the expression of *nis*F, *nis*E

and *nis*G genes clearly reduced immunity showing that this transcript is also involved in nisin immunity (Siegers and Entian, 1995).

In some cases, the regulatory systems involved in nisin biosynthesis and the presence of nisin in the culture supernatant can influence nisin production and immunity. Dodd et al. (1996a) demonstrated that the response regulator protein, NisR, is essential for inducing immunity of a wild-type cell. The ability to induce full immunity could result in the ability to achieve maximum production of nisin.

In addition to acting as an antibacterial peptide, nisin also has the ability to act as a signal peptide to induce its production (Kleerebezem et al., 1999). The NisK protein senses nisin in the culture medium and disruption of the *nis*K gene results in a cell that is no longer inducible by nisin (Kuipers et al., 1995). NisK is involved in the phosphorylation of the NisR protein. The NisR protein regulates gene expression by activating the transcription of the nisin gene cluster. Of the three identified promoters preceding *nis*A, *nis*R and *nis*F, the *nis*A and *nis*F promoters are nisin-inducible (de Ruyter et al., 1996).

In summary, the expression of the nisin gene cluster is regulated by a two-component nisin-inducible system that consists of the regulatory proteins, NisK and NisR. The nisin precursor molecule undergoes modification by NisB and NisC proteins, and it is translocated across the cell membrane by the NisT protein. The leader peptide is cleaved by the NisP protein resulting in a mature nisin molecule. The NisI, NisF, NisE and NisG proteins protect the producer cell against its activity. Advancements in the understanding of the genes involved in the biosynthesis of nisin have resulted in opportunities for protein engineering of nisin.

1.5.2. *Protein engineering of the nisin molecule.*

The fact that nisin is generated from a precursor peptide allows the opportunity to use protein engineering to process variant nisin molecules. Dodd et al. (1995) described a cassette vector to facilitate mutagenesis of the entire *nis*A structural gene. Similarly, Dodd et al. (1996b) described a gene replacement strategy to produce variant nisins in which gene replacement was used to incorporate a copy of the chromosomally-located *nis*A gene into a host strain containing all other genes required for nisin biosynthesis. This process enabled variant nisin genes to be expressed from its natural loci in place of the chromosomal wild-type structural gene. The ability to make specific changes in a nisin molecule by site-directed mutatgenesis has provided great insight into the mode of action of nisin.

1.5.3. *Mode of action of the nisin molecule.*

1.5.3.1. *Spectrum of activity.* Nisin is considered to have a narrow spectrum of activity because it does not inhibit gram-negative bacteria, yeasts or fungi (Hurst, 1981). However, the growth of a broad spectrum of gram-positive bacteria is inhibited by nisin. Early studies showed that nisin inhibited other streptococci, aerobic spore-formers and other gram-positive bacteria that are associated with the spoilage of milk products (Kalra and Dudani, 1975). Results of antibacterial assays of nisin A and Z against the vegetative cells of various strains of *Listeria*, *Clostridium*, *Staphylococcus*, *Lactococcus*, *Lactobacillus*, *Micrococcus*, and *Pediococcus* showed that the minimum inhibitory concentrations varied considerably between species and between strains of the same species (Meghrous et al., 1999).

Comparison of the two naturally-occurring nisin variants (A and Z) showed no difference in biological activity (de Vos et al., 1993). Although larger inhibition zones were observed in activity assays for the undiluted supernatant of a nisin Z producer, the minimum inhibitory concentrations were the same for nisin Z and nisin A. In this controlled study, it was assumed that the increase of zone size is the result of a better diffusion rate, perhaps because of the more polar asparagine instead of histidine at position 27.

The inability of nisin to inhibit the growth of gram-negative bacteria is due to the protective outer membrane that covers the cytoplasmic membrane and peptidoglycan layer of the cells. The outer membrane acts as a barrier that excludes hydrophobic substances and macromolecules; therefore, nisin is unable to reach its target of action (Helander and Mattila-Sandholm, 2000). Studies have shown that the introduction of permeabilization agents, such as chelating agents, disrupt the outer membrane and render nisin active against gram-negative bacterial cells.

1.5.3.2. *Structure-function activity relationships*. The structure of nisin, as shown in Figure 1.1A, was first reported by Gross and Morell (1971). It is a 34-amino acid peptide consisting of one lanthionine (3-7), four β-methyllanthionines (8-11, 13-19, 23-26, and 25-28), one dehydrobutyrine (2) and two dehydroalanine residues (5 and 33). NMR analysis revealed that the structure of nisin consists of an amphiphilic N-terminal fragment (3-19) joined by a flexible hinge region around Met21 to a rigid double ring fragment (23-28; van de Ven and Jung, 1996). Early studies on the biological activity of

nisin showed that cleavage of the C-N bond of Dha in nisin resulted in loss of activity against *Staphylococcus aureus* (Gross and Morell, 1967).

Analysis of pure and commercial nisin demonstrated that a number of naturally-produced degradation products of nisin are present (Rollema et al., 1996; Cruz et al., 1996). Analysis of nisin by capillary electrophoresis, MALDI-TOF MS and electrospray ionization suggested that all degradation occurs at either the carboxy terminal of the peptide or in the A ring (Cruz et al., 1996). It appears that the Dha residues are susceptible to an acid-catalyzed addition of a water molecule to the double bond (Rollema et al., 1996). This results in the formation of a 2-hydroxy residue in which the polypeptide bond is cleaved into an N-terminal peptide amide and a C-terminal pyruvyl peptide, as shown in Figure 1.6. This reaction results in the formation of two chemically-modified species: [nisin1-32]-peptide amide and [Ile-4-amide, pyruvyl-Leu-6]*des*-Dha5-nisin(1-32) peptide amide. Table 1.2 outlines these and other known degradation products of nisin.

Table 1.2. Known degradation products of nisin.

Compound	Molecular mass	Reference
[2-hydroxy-Ala5] nisin	3369.6	Rollema et al., 1996
[Ile-4-amide, pyruvyl-Leu6]*des*-Dha5-nisin	3369.6	Rollema et al., 1996
[Ser33] nisin	3369.6	Rollema et al., 1996 Chan et al., 1996
[Met(O)21] nisin	3367.5	Rollema et al., 1996
Nisin 1-32	3153.5	Rollema et al., 1996 Chan et al., 1996
[2-hydroxy-Ala5] nisin 1-32 peptide amide	3171.5	Rollema et al., 1996
[*des*-Dha5] nisin 1-32 amide	3174.89	Chan et al., 1996

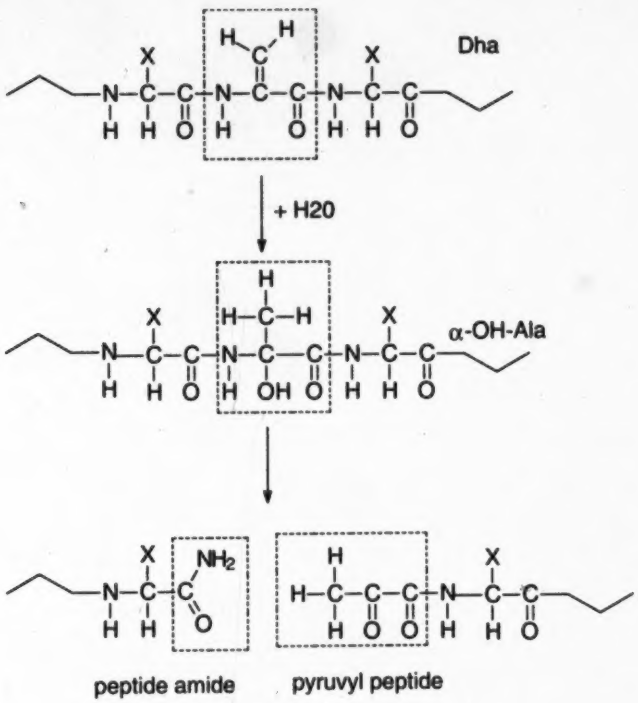

Figure 1.6. The chemical reaction of an acid-catalyzed addition of a water molecule to a dehydroalanine molecule resulting in the cleavage of a peptide bond and the formation of a peptide amide and a pyruvyl peptide.

Most compounds listed in Table 1.2 show similar activity to the parent nisin molecule, except for a strong decrease in antibacterial activity with [2-hydroxy-Ala5] and [Ile-4-amide, pyruvyl-Leu6]*des*-Dha5 nisin (Rollema et al., 1996). This illustrates the importance of dehydroalanine at position 5 and the integrity of the first ring of the nisin molecule for its activity.

Proteolytic cleavage of the nisin molecule also helps define which regions are essential for biological activity. Cleavage products are shown in Table 1.3. Results of activity assays of the cleavage products revealed that the C-terminus tail of nisin played a

significant role in the activity of nisin. Cleavage or removal of Ala15-Leu16 in ring C resulted in complete loss of activity (Chan et al., 1996). Nisin 1-12, which lacks ring C, is also inactive. Results also showed that an intact ring A is essential for activity.

Table 1.3. Detected compounds after proteolytic cleavage of nisin (from Chan et al., 1996).

Compound	Molecular mass
Nisin	3355.1
Ser[33] nisin	3373.1
Nisin 1-32 amide	3156.9
Nisin 1-31	3058.8
Nisin 1-29	2808.5
Nisin 1-20	1881.3
[Ala15-OH, H-Leu16] nisin 1-29	2826.5
[*des*-Leu16, Ala15-OH, H-Met17] nisin 1-29	2713.3
Nisin 1-12	1151.4
[*des*-Dha5] nisin 1-32 amide	3174.9

1.5.3.3. *Site-directed mutagenesis of nisin*. Site-directed mutagenesis has allowed for the production of a number of variant molecules of nisin. This method of engineering has been essential in elucidating the regions of nisin that are important for antibacterial activity.

Serine codons in the DNA of the precursor peptide were changed to alanine codons to prevent the dehydration of serine to dehydroalanine (Dodd et al., 1995). Lactococcal host strains were successfully transformed, containing the genetic information for either [Ala5]-nisin, [Ala33]-nisin or [Ala5,33]-nisin. All transformants displayed antibacterial activity indicating that the Dha residues are not exclusively involved in nisin activity. When Dha5 was changed to an alanine residue, a loss of

approximately 50% in activity compared with the wild-type strain was observed; however, it is not known whether this loss of activity was a result of a less efficient molecule, poor processing or secretion, or a reduction in gene expression (Dodd et al., 1995). The insertion of Dhb instead of a Dha residue at position 5 also resulted in a loss of antibacterial activity, but the variant compound was more resistant to acid-catalyzed chemical degradation at that site (Rollema et al., 1995). Although [Ala5] nisin still exhibited activity against vegetative cells, it was much less active against the outgrowth of spores of *Bacillus subtilus* (Chan et al., 1996).

The C-terminal region of nisin has also been studied to determine its role in activity. Three nisin variants were produced with the Val32 residue replaced by either a Glu, Lys or Trp residue (van Kraaij et al., 1997). All three of the mature nisin molecules were transcribed with a serine residue at position 33 instead of the usual Dha. [Trp32,Ser33] nisin, [Glu32,Ser33] nisin and [Glu32] nisin 1-32 showed a 3 to 5 fold loss in activity, whereas [Lys32,Ser33] nisin exhibited similar activity to the wild-type, indicating that negative charges in the C-terminus part of nisin resulted in loss of activity. The importance of ring C in the antibacterial activity of nisin was demonstrated by designing a mutation in which Thr13 was replaced by a cysteine residue (van Kraaij et al., 2000). This mutation prevented the formation of the thioether bond of ring C and resulted in an intramolecular disulfide bond between Cys13 and Cys19. The activity of the variant nisin molecule was reduced to <1% of that of nisin.

1.5.3.4. *Mode of action against vegetative cells.* Nisin appears to target the cytoplasmic membrane of vegetative cells. The cytoplasmic membrane is composed primarily of

proteins and phospholipids, and because there are no antagonistic effects against other proteins, phospholipids are the likely membrane constituents to interact with nisin (Henning et al., 1986b). The apolar nature of several amino acids of nisin make hydrophobic interactions between the amino acids and carbon chains of the fatty acids likely. It was also suggested that electrostatic interactions may occur between the ionic groups of nisin and the phosphate groups of the cytoplasmic membrane (Henning et al., 1986b).

The common mechanism of action of nisin is the dissipation of the membrane potential ($\Delta\psi$) and pH gradient of the cell, which results in a total collapse of the proton motive force. It appears that nisin first interacts with phospholipids on the cell membrane and permeabilizes target cells. This is followed by the leakage of low molecular weight cellular compounds, degradation of vital macromolecules, inhibition of biological processes, and cell lysis (Bruno et al., 1992; Montville and Bruno, 1994; Abee et al., 1994; Winkowski et al., 1996).

Evidence of nisin interaction with the cell membrane has been demonstrated with the use of synthetic membranes and membrane components. The affinity of nisin for anionic lipids was reported by Driessen et al., (1995) and Demel et al. (1996). In the presence of anionic phospholipids, nisin forms non-selective, transient pores in cells, proteoliposomes, liposomes and black lipid membranes (Moll et al., 1996). It was suggested that the cationic nisin molecule is associated with the anionic surface of peptidoglycan liposomes resulting in pore formation (Driessen et al., 1995).

As described above, nisin contains two structured domains: the N-terminus domain (3-19) and the C-terminus domain (22-28). The C-terminus domain contains a

major part of the positively charge of the nisin molecule; therefore, it is likely that this domain interacts with the membrane surface. A mutant containing the negatively-charged glutamic acid at position 32 was created, and the presence of a negative charge in the C-terminus abolished the lipid dependency of the nisin-membrane interaction (Breukink et al., 1997). The C-terminus region of nisin also plays a role in nisin activity by translocating across the cell membrane to form pores (van Kraaij et al., 1998).

Later studies determined the interaction between nisin and lipid II. Lipid II is a cell wall precursor that has a primary function of transporting the pentapeptide disaccharide building blocks of peptidoglycan synthesis, prefabricated in the cytoplasm, to their site of polymerization (Delcour et al., 1999). Mersacidin, a type B lantibiotic, binds to the lipid II molecule to inhibit bacterial growth (Brötz et al, 1998a). It was shown that nisin, a type A lantibiotic, also interacts with lipid II to promote pore formation, and subsequently cell death (Brötz et al., 1998b). It was suggested that the high affinity of nisin for lipid II, which likely involves an interaction with the N-terminal rings of nisin, and its pore-forming ability results in its high level of activity (Breukink and de Kruijff, 1999; Breukink et al., 1999). The ability of nisin to use lipid II as a docking molecule for activity is decreased with mutations in the N-terminus region of nisin (Wiedemann et al., 2001). Furthermore, total activity is also decreased, with complete inhibition of pore formation, with a mutation in the flexible hinge region (20-21; Wiedemann et al., 2001). This research demonstrates that nisin's high level of activity is the result of two killing mechanisms as demonstrated in Figure 1.7: 1) the interaction of the N-terminus domain with lipid II molecule that inhibits peptidoglycan synthesis of the cell and 2) translocation

of the C-terminus domain across the cell membrane to form pores and dissipate the proton motive force.

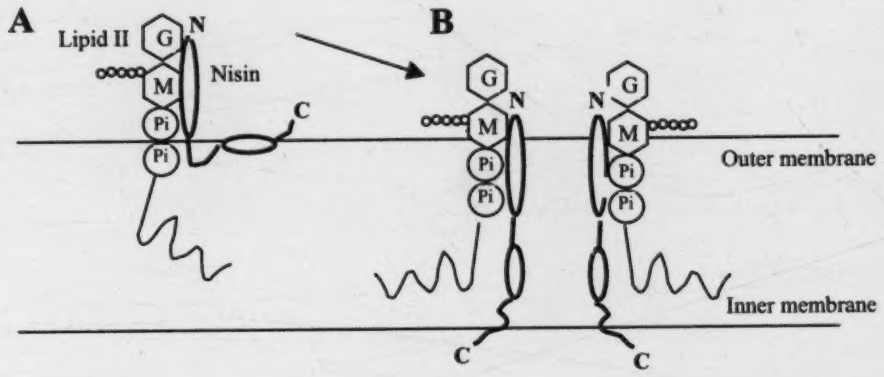

Figure 1.7. Model of antibacterial activity of nisin showing two distinct mechanisms of action: A) interaction of nisin with lipid II molecules can inhibit peptidoglycan synthesis and B) results in pore formation after the C-terminus region is translocated across the cell membrane. Based on Wiedemann et al. (2001).

1.5.3.5. *Mode of action against bacterial spores*. The sporicidal activity of nisin against spores of *Clostridium botulinum* was examined by Scott and Taylor (1981a). Studies in TPYG broth showed that type E spores were more sensitive to nisin than type B and that type A spores were the most resistant. Nisin at 50 to 1000 IU/mL was sufficient to inhibit the outgrowth of type E spores, whereas 200 to 2500 IU/mL was needed to inhibit type B and type A spores. The same concentrations of nisin were less effective in inhibiting spore outgrowth in cooked meat medium for unknown reasons. It was speculated that nisin binds to meat particles. Further studies examined the effects of pH and temperature on the ability of nisin to prevent spore outgrowth (Scott and Taylor, 1981b; Somers and

Taylor, 1981). In all cases, type E spores were the most sensitive and type A spores were the most resistant to the effects of nisin. It was determined that the antibotulinal effectiveness of nisin is greater at pH levels of 5.5 to 6 compared with pH 7 or 8 (Somers and Taylor, 1981). This is could be either due to the increased solubility of nisin at a more acidic pH or an acid-damaging effect on the bacterial spores. Nisin is also more effective at inhibiting the outgrowth of spores following a heat-damaging treatment (Scott and Taylor, 1981b). It seems that the effectiveness of nisin on spores is dependent on a number of conditions, including: the temperature of heat-shock treatment, the length of the heating period, pH and spore load. It also appears that specific nutrients may be required to sensitize spores to heat. Studies indicated that the addition of a commercial preparation of nisin, Nisaplin (containing 2.5% nisin), to dairy-based products intended to be heated actually decreased the level of thermal resistance of selected bacterial spores (Beard et al., 1999).

The mode of action of nisin against spores is suggested to be through a reaction with a sulfhydryl (-SH) group that is associated with the membrane of freshly germinated spores. Early studies suggested that the addition of mercaptans is required for the biological action of nisin (Gross and Morell, 1967). These –SH groups can be covalently modified to cause inhibition of spore outgrowth (Morris et al., 1984). Nisin meets the criteria of a good sulfhydryl agent as it is an agent of moderate reactivity in a relatively bulky, charged molecule (Morris et al., 1984). Nisin also acts as a sulfhydryl agent because it has dehydro residues that can act as potential Michael acceptors to react with sulfhydryl groups. Morris et al. (1984) showed that nisin reacts with several sulfhydryl-containing molecules and that this activity is associated with the dehydro residues. This

was also later demonstrated by Liu and Hansen (1990) who looked at nisin reactivity with simple mercaptan molecules. NMR results showed the loss of proton signals from all three dehydro residues.

1.5.4. *Applications of the bacteriocin nisin to foods.*

Nisin is the only bacteriocin licensed for use in foods and it is approved for use in over 50 countries. It has achieved successful application in several dairy products, canned foods, and vegetables (Delves-Broughton et al., 1996). To be considered as a potential food additive, a bacteriocin must: 1) be non-toxic to humans; 2) cause no change in the physical or chemical properties of the food; 3) be stable during storage; 4) be effective at low pH; 5) serve no medical purpose, and 6) be inexpensive (Oyarzabal, 1998). The successful application of nisin in foods requires the special properties of nisin to be considered. For example, the pH of the food should be less than seven to ensure solubility and stability of nisin (Henning et al., 1986a). Various food components also contribute to the effectiveness of nisin. With increasing temperature and phospholipid and protein concentration, nisin lost or exhibited a delay in its antibotulinal effectiveness (Rogers and Montville, 1994).

The potential for the use of nisin in foods was summarized by Fowler (1979) showing that nisin controlled the growth of *C. botulinum* in spreadable cheese products and canned soups and vegetables. Nisin also had potential as a preservative agent in other dairy products, such as milk and spreads containing milk products. Nisin was shown to be an adequate antibacterial agent for use in pasteurized egg whites and liquid whole eggs (Niewiarowicz, 1981; Calderon-Miranda et al., 1999). The addition of nisin has also

prevented malolactic fermentation in wines (Daeschel, 1989) and the growth of *Bacillus cereus* in crumpets (Jenson et al., 1994). Although the addition of nisin has enjoyed success in these foods, nisin has had limited success in meat products.

1.5.4.1. *Application of nisin to meat products*. The application of nisin as a preservative in fresh meat products has been ineffective except when used in high concentrations (Scott and Taylor, 1981b; Rayman et al., 1983; Bell and de Lacy, 1986; Chung et al., 1989; Stevens et al., 1991; Mahadeo and Tatini, 1994). The use of nisin in meat products appears to be limited to heat-treated meat products or in conjunction with other preservatives. Early studies examining the antibotulinal effectiveness of nisin failed to demonstrate inhibition of spore outgrowth in meat slurries (Rayman et al., 1983; Taylor et al., 1985). Studies in bacon showed that nisin is only an effective antibotulinal agent when used in combination with nitrite (Taylor and Somers, 1985). Other studies demonstrated an initial inhibitory effect on various spoilage and foodborne pathogens after the addition of either pure nisin, a commercial nisin preparation or a nisin-producing strain of *L. lactis* to meat; however, after storage, little differences in microbial growth between the test and control samples was noted (Wang et al., 1986; El-Khateib et al., 1993; Murray and Richard, 1997; Pawar et al., 2000).

The application of nisin to cooked meats had greater success than in fresh meats. The addition of nisin to pork tenderloins cooked at 121°C for 15 minutes and inoculated with *L. monocytogenes* prevented bacterial growth (Fang and Lo-Wei, 1994). The shelf-life of heat-treated bologna sausages was extended to >50 days after treatment with nisin (as Nisaplin), compared with 7 days for the control (Davies et al., 1999). Nisin, in

combination with other additives (e.g., sorbate, organic acids) or processing mechanisms (e.g., ultra high pressure, modified atmosphere packaging), has also resulted in significant reductions in bacterial growth (Avery and Buncic, 1997; Yuste et al., 1998; Ariyapitipun et al., 1999; Barbuddhe et al., 1999; Ariyapitipun et al., 2000)

The failure of nisin in fresh meat was suggested to be the result of proteolysis of nisin by meat enzymes, binding of nisin to meat proteins, poor solubility of nisin and uneven distribution of nisin (Rayman et al., 1983; Taylor and Somers, 1985; Chung et al., 1989; Cutter and Siragusa, 1996; Cutter and Siragusa, 1998). The understanding of the interaction of nisin in complex food matrices, such as meats, is hampered by inefficient and insensitive detection methods.

1.6. Detection methods for bacteriocins.

Traditional methods of detecting bacteriocin activity are based on agar diffusion assays (such as spot or well diffusion; Parente et al., 1995; Wolf and Gibbons, 1996). Such methods are indirect because they rely on the choice of a sensitive indicator organism, which differs between laboratories (McMullen and Stiles, 1996). As well, the results are expressed in arbitrary units, which vary with experimental conditions (Bouksaim et al., 1998). Several methods have since been described for the detection and quantification of nisin.

Relatively pure nisin can be detected using a variety of methods. Ward et al. (1994) distinguished between nisin A and Z variants using a ligase chain reaction that is a sensitive and specific method for differentiating similar DNA sequences. Fourier transform ion cyclotron resonance and electrospray ionization mass spectrometry were

used to analyze nisin and some of its variants and degradation products (Lavanant et al., 1998a; b). Other methods detected nisin in complex media. The generation of antibodies allowed the development of ELISA protocols to detect nisin in the supernatant of producer strains (Suárez et al., 1996; Bouksaim et al., 1999). Capillary zonal electrophoresis detected nisin in milk after a simple extraction procedure to remove caseins and lipids, which interfered with nisin detection (Rossano et al., 1998). Bioluminescence methods were also developed for the detection of nisin in milk (Wahlström and Saris, 1999).

The study of the interaction of bacteriocins with food components requires a detection method that is not limited by complex food matrices. A sensitive, rapid detection method for bacteriocins could be a useful method to track purification procedures, to detect bacteriocin production in experiments involving genetic manipulation and to detect bacteriocins in foods (McMullen and Stiles, 1996; Bouksaim et al., 1998). Detecting the bacteriocin by means of searching for a compound with the appropriate molecular weight is one method of confirming the presence of bacteriocins in fully-grown bacterial cultures or food products. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has potential as one such method.

1.6.1. *MALDI-MS.*

MALDI-MS was first introduced in 1988 by Karas and Hillenkamp (1988) as a rapid and simple method for detection of proteins with a molecular mass greater than 10 000 Da. Combining the analyte with a matrix compound that exhibited a strong absorption at the specific wavelength of the ultraviolet laser allowed for the detection of

intact, high molecular weight proteins. The ability of this technique to detect nonvolatile bioorganic compounds, at subnanogram concentrations, has greatly advanced the field of mass spectrometry.

Early mass spectrometry methods using laser irradiation often resulted in destruction of the sample (Beavis, 1991). The principle of MALDI-MS involves a low concentration of analyte molecules being embedded in a matrix and spotted on a probe. Pulsed UV laser beams then desorb and ionize the co-crystallized sample/matrix from the probe surface, as illustrated in Figure 1.8. The matrix absorbs the laser energy and minimizes sample damage (Siuzdak, 1994). The matrix molecules emit the absorbed energy in the form of heat that causes sublimation of the matrix crystals and expansion of the matrix and the analyte into the gas phase (Yates, 1998).

MALDI-MS has many advantages over other forms of mass spectrometry with the most significant being the ability to analyze molecules in complex mixtures. A list of the advantages and disadvantages of MALDI-TOF MS are given in Table 1.4.

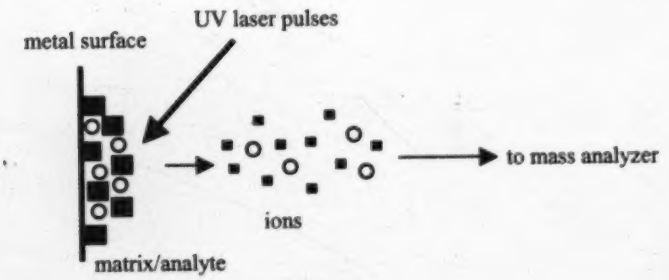

Figure 1.8. Diagram showing the MALDI source of ions. Pulsed laser beams generate analyte ions by desorbing them from a matrix material resulting in the fragmentation of the matrix (■) material with intact analyte (○) ions.

Table 1.4. Advantages and disadvantages of MALDI-MS (Adapted from Siuzdak, 1994).

	Mass limit, Da (practical)	Advantages	Disadvantages	Suitable compounds
MALDI-MS	Theoretically unlimited (<400 000)	-Tolerant of low concentrations of salts -High mass capability -Tolerant of mixtures and impurities -Femtomole sensitivity -Ability for sequence analysis -Low sample consumption -Ease of preparation -Short measurement time	-Typically low mass resolution ($m/\Delta m$ = 100-400) -Mass accuracy of ±0.1%-0.01% -Not amenable to LC/MS	-Peptides -Proteins -Glycoproteins -Carbohydrates -Nucleotides -Oligonucleotides -Phosphoproteins -Small chargeable molecules -Heterogenous samples

1.6.1.1. *Instrumentation*. MALDI-MS is typically used with a time-of-flight (TOF) mass analyzer. This method measures the mass of an ion using its velocity to determine the mass-to-charge ratio (Hillenkamp et al., 1991). Ions are first accelerated to a fixed kinetic energy by the application of an electric potential. Then they pass through a field-free region where they travel at a speed relative to their mass. A detector at the end of this region produces a signal as each ion strikes it. According to the theory of kinetic energy ($KE = \frac{1}{2}mv^2$), smaller ions will travel at a higher velocity and reach the detector before larger ions. The mass range for TOF analyzers is theoretically unlimited; however, there are certain limitations because of sample behavior and the detector response (Costello, 1997). This sets the practical detection limit at 1 000 000 Da. Furthermore, realistic measurements have only been made for compounds less than 400 000 Da.

There are two types of TOF analyzers, as shown in Figure 1.9. The more basic type is a linear instrument where the ions travel in a straight path to reach the detector. The second is a reflectron analyzer in which an ion mirror is placed in the path of the

ions. The reflectron analyzer is typically more sensitive and results in better mass resolution than the linear instrumentation.

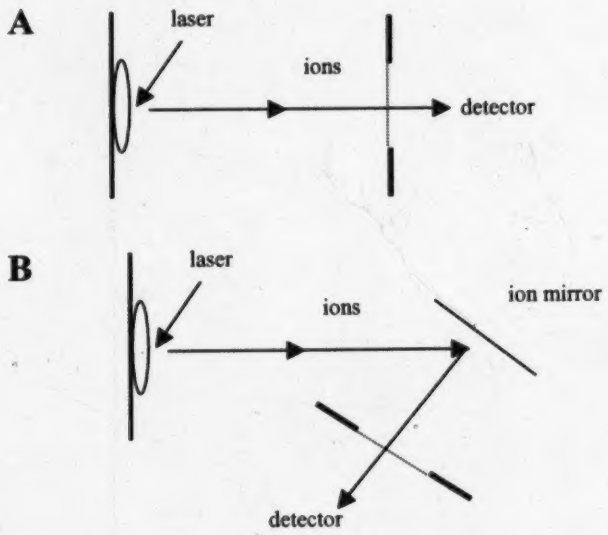

Figure 1.9. Diagram of A) linear and B) reflectron MALDI-TOF mass spectrometers. Based on Hillenkamp et al. (1991).

1.6.1.2. *Sample preparation.* Sample preparation is thought to be the most crucial procedure in the analysis of biological samples by MALDI-TOF MS. Sample preparation generally involves two steps: 1) the isolation of the analyte molecule which is relatively free of contaminants, and 2) the sample processing that involves the choice of matrix and analyte concentration, pH adjustment, crystallization conditions, and on-target sample clean-up (Kussmann et al., 1997).

Three general methods exist for depositing the sample on the probe for analysis (Kussmann et al., 1997). The dried-droplet method involves mixing the analyte and matrix (1:1), spotting the mixture on the probe, and allowing it to air dry. The thin-layer method involves first spotting a matrix layer onto the probe. After it is dry, the analyte is spotted onto the matrix layer. The two-layer (sandwich) method is performed in the same initial manner as the thin-layer method; however, a mixture of the analyte and matrix (1:1) is spotted onto the matrix layer. Although the two-layer method is particularly effective for analysis of complex peptides and proteins (Dai et al., 1999), there is no one method of sample preparation that works for all analyses.

Success is highly dependent on the crystallization of the matrix-analyte mixture. Therefore, selection of the proper matrix compound is essential. The matrix functions to absorb the energy of the laser light and to isolate the biopolymer molecules from each other (Hillenkamp et al., 1991). A good matrix for a given sample must: 1) desorb the laser energy at the wavelength employed; 2) be soluble in the same solvent as the analyte material; 3) have physical properties such as lattice structure and heat of sublimation to induce efficient desorption and promotion of ionization, and 4) have good vacuum stability (Muddiman et al., 1995).

Analysis of proteins by MALDI-TOF MS typically involves co-crystallizing the analyte with a matrix that includes a cinnamic acid or hydroxylated benzoic acid derivative (Roepstorff, 2000). Sinapinic acid is often used because of its tolerance of high concentrations of salts and chaotrophic agents in the sample (Beavis, 1991).

1.6.1.3. *Improvements in mass resolution and accuracy.* Mass resolution (m/Δm) is a measure of the instrument's ability to produce separate signals from ions of a similar mass (Hillenkamp et al., 1991). It is determined by dividing the mass of a given signal (m) by the full width of the signal (Δm) that is measured between the points at ½ the maximum intensity as shown in Figure 1.10. Whereas, mass accuracy is a measure of error involved in assigning a mass to a given ion signal (Hillenkamp et al., 1991). This is expressed as the ratio of the mass assignment error divided by the mass of the ion.

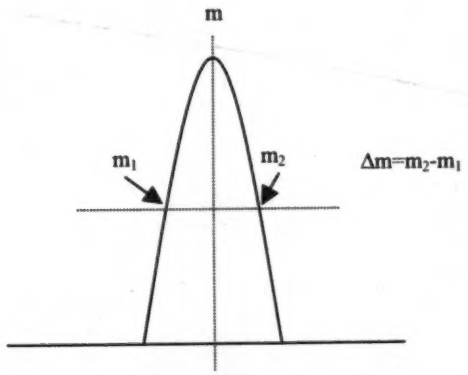

Figure 1.10. Determination of mass resolution (m/Δm) in mass spectrometry.

MALDI-TOF MS instruments have greatly improved mass resolution due to an advancement called pulsed ion (or delayed) extraction or time-lag focusing. As mentioned above, early MALDI-TOF MS instruments applied an electric potential upon ion formation. This caused ions of the same mass-to-charge ratio (m/z) to arrive at the detector at a slightly different times, thus resulting in poor resolution. The principle of pulse ion extraction, as shown in Figure 1.11, allows the ions to first expand into a field free region to separate according to their initial velocities (Whittal and Liang, 1997).

After a time delay, an electric potential is applied to extract the ions into the flight tube. This results in improved resolving power of both linear and reflectron instruments, as well as an improvement in mass measurement because peak heights can be better distinguished from the background noise (Whittal and Liang, 1997). Delayed extraction provides improved mass resolution and mass accuracy in proteins with molecular weights less than 30 000 Da (Whittal et al., 1997; Barbacci et al., 1997; Amft et al., 1997; Bahr et al., 1997).

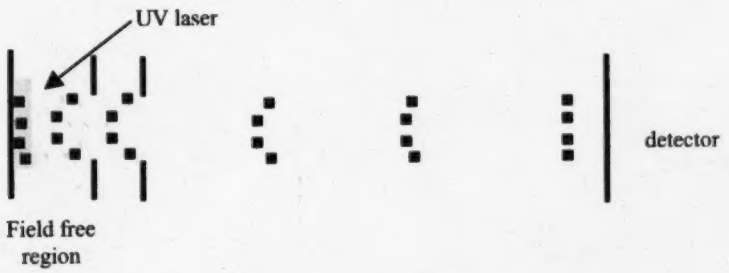

Figure 1.11. Schematic diagram illustrating the pulsed ion extraction technology.

1.6.1.4. *Applications in analysis of proteins and peptides.* MALDI-TOF MS is a powerful tool in the analysis of peptides and proteins, particularly from biological sources, because of its ability to tolerate the presence of contaminants that are often associated with the isolation of biomolecules. Biological preparations typically involve the use of non-volatile salts, chaotrophic agents, preservatives and/or detergents that typically interfere with mass spectrometric analysis (Dalluge, 2000). The most recognized functions of MALDI-TOF MS are molecular weight determination and structural elucidation of peptides and proteins (Gevaert et al., 1997). MALDI-TOF MS analysis

may result in fragmentation of the analyte molecule. In such a situation, the parent ion and its daughter fragments separate and yield peaks with different masses (Jimenez and Burlingame, 1998). This phenomenon, called post-source decay, has been used to obtain incomplete sequence information of small- to moderate-sized peptides (Reiber et al., 1998; Chaurand et al., 1999).

Various studies using MALDI-TOF MS for the analysis of peptides and proteins have resulted in methods for the extraction of proteins from polyacrylamide gels (Ehring et al., 1997), extraction of proteins from tissue sections (Caprioli et al., 1997), determination of the relationship between peptide structure and signal intensity (Wenschuh et al., 1998), and the identification of bacteria based on specific biomarkers (Liang et al., 1996; Krishnamurthy et al., 1996; Holland et al., 1996; Wang et al., 1998; Welham et al., 1998; Demirev et al., 1999; Arnold et al., 1999; Holland et al., 1999).

1.7. Research objectives.

Although the use of MALDI-TOF MS in the analysis of peptides and proteins has received a great deal of attention, little research has applied the use of MALDI-TOF MS to the analysis of bacteriocins or of food components (Sporns and Abell, 1996). It was recognized that MALDI-TOF MS had the potential for use in the detection of bacteriocins from complex mixtures, including food matrices. Therefore, the objective of this study was to investigate the possibility of using MALDI-TOF MS for bacteriocin detection. Successful application of this technique could then be applied to study the interaction of bacteriocins with food components. The primary objectives of this work were:

1. To develop a method using MALDI-TOF MS for the detection of bacteriocins in bacterial culture supernatants.
2. To use MALDI-TOF MS to determine the fate of bacteriocins throughout a purification.
3. To use MALDI-TOF MS to study the fate of nisin in meat.

The study of the fate of nisin in meat showed that nisin reacts enzymatically with a compound of a molecular weight of 307 Da. This compound was hypothesized to be glutathione. To confirm this hypothesize, a number of studies were designed:

1. To isolate glutathione S-transferase from meat and partially characterize its properties.
2. To examine the *in vitro* reaction between glutathione and nisin.
3. To determine the site(s) of reaction between glutathione and nisin.

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CHAPTER 2*

DETECTION OF BACTERIOCINS USING MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY.

2.1. Introduction.

Bacteriocin-producing lactic acid bacteria, partially purified bacteriocins and purified bacteriocins have the potential for use as natural biopreservatives to extend storage life and to enhance the safety of foods. Currently, researchers rely on bioassays such as deferred inhibition and agar diffusion tests to detect the presence of bacteriocin production in foods (McMullen and Stiles, 1996). Such methods are indirect because they rely on a sensitive indicator organism that varies between laboratories. As well, the results are expressed in arbitrary units, which vary with experimental conditions (e.g., pH, temperature, nutrients, and choice of indicator) (Jack et al., 1995; Bouksaim et al., 1998). A sensitive, rapid detection method for bacteriocins could be a useful method to track purification procedures, to detect bacteriocin production in experiments involving genetic manipulation and to detect bacteriocins in foods (Bouksaim et al., 1998; McMullen and Stiles, 1996). Detecting the bacteriocin by means of searching for a compound with the appropriate molecular weight is one method of confirming the presence of bacteriocins in fully-grown bacterial cultures or food products. MALDI-TOF MS appears to have potential as one such method.

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With rapid advances in this technology, MALDI-TOF MS is becoming a valuable tool in the analysis of biopolymers and is often used for molecular weight measurements. MALDI-TOF MS has also been used routinely for the analysis of synthetic peptides and proteins. However, the analysis of peptides and proteins extracted from biological sources has been hampered by the presence of contaminants such as salts, glycerol, and detergents (Amado et al., 1997; Blackledge and Alexander, 1995a,b; Kussmann et al., 1997; Worrall et al., 1998; Yates, 1998). It has been shown that sodium dodecyl sulfate and salts interfere with the signals obtained for BSA and cytochrome c (Amado et al., 1997; Wang et al., 1998). Wang et al. (1998) reported that differences in salt content among samples might result in detection of certain peptides and proteins over others, yielding different mass spectral patterns and consequently poor spectrum reproducibility. These contaminants may completely suppress the peptide and protein signals.

In bacteriocin purification, the presence of detergents such as Tween in APT and Lactobacillus MRS media, both commonly used for the growth of bacteriocin-producing organisms, may interfere with the analysis. It is suggested that Tween 80 is an essential media component for bacteriocin production and detection (Huot et al., 1996; Joosten and Nũnez, 1995). It is likely that partial purification of bacteriocin preparations will be necessary to obtain a clean sample for analysis using MALDI-TOF MS; however, purification methods are often time-consuming, result in the loss of biological material and may even introduce more contaminants that are incompatible with MALDI-TOF MS (Worrall et al., 1998).

Researchers have investigated the use of activated synthetic membranes as an alternative to a stainless steel sample support in MALDI-TOF MS analysis. By spotting a

sample onto a membrane such as polyethylene, contaminants can be washed away leaving the peptides and proteins intact at the surface for analysis (Blackledge and Alexander, 1995a,b; Worrall et al., 1998). This process resulted in equal or greater sensitivity and mass resolution for all samples compared with those desorbed from stainless steel. Synthetic membranes were particularly suitable for high mass molecules (e.g., >30 000 Da) because severe ion suppression is typically observed in the analysis of high mass mixtures (Blackledge and Alexander, 1995a,b). Other researchers have used or suggested further processing of the sample on the probe, such as a wash with cold water to remove the contaminants (Kussmann et al., 1997; Wang et al., 1998; Yates, 1998).

In this study, a MALDI-TOF MS method was developed for the detection of bacteriocins in culture supernatant, and the method was used to determine the fate of a bacteriocin throughout a purification procedure.

2.2. Materials and Methods.

2.2.1. *Bacterial strains and media.*

The bacteriocin-producing lactic acid bacteria used in this study are listed in Table 2.1. *Enterococcus faecium* CTC 492 was obtained from Dr. M. Hugas (Institut de Recera i Technologia Agroalimentàries, Girona, Spain) and *E. faecium* BFE 900 was isolated from black olives by Franz et al. (1996). *Carnobacterium divergens* LV 13, obtained from Dr. B. G. Shaw (Institute of Food Research Langford, Bristol, UK), and *Lactobacillus sakei* DSM 20017 were used as sensitive indicator organisms against the producer strains. Frozen stock cultures were maintained at -70°C in Bacto APT broth

(Difco Laboratories, Detroit, Michigan) supplemented with 20% glycerol (v/v). Prior to experimental use *L. lactis* ATCC 11454, *Brochothrix campestris* ATCC 43754, *Pediococcus acidilactici* PAC-1.0, *E. faecium* CTC 492 and BFE 900 and *C. divergens* LV 13 cultures were subcultured twice and grown overnight in APT broth. *L. sakei* DSM 20017 was subcultured twice and grown overnight in Lactobacillus MRS broth (Difco) prior to use. Solid agar media were prepared by adding 1.5% (w/v) granulated agar (Difco) to either APT or Lactobacillus MRS media. Soft APT and MRS agar were prepared with 0.75% agar (w/v).

Table 2.1. Bacteriocin–producing organisms used in this study and characteristics of their bacteriocins.

Producer Strain	Bacteriocin	Amino Acids	MW (Da)	Reference
L. lactis subsp. *lactis* ATCC 11454	nisin	34	3354	(Gross and Morell, 1971)
B. campestris ATCC 43754	brochocin A	59	5242	(McCormick et al., 1998)
B. campestris ATCC 43754	brochocin B	43	3943	(McCormick et al., 1998)
P. acidilactici PAC-1.0	pediocin PA-1	44	4629	(Henderson et al., 1992)
E. faecium CTC492	enterocin A	47	4829	(Aymerich et al., 1996)
E. faecium BFE900	enterocin B enterocin B enterocin A	53	5463	(Franz et al., 1999)

2.2.2. *Preparation of culture supernatant.*

L. lactis ATCC 11454, *P. acidilactici* PAC-1.0, *B. campestris* ATCC 43754, *E. faecium* CTC 492 and *E. faecium* BFE 900 were grown for 18 h at 30°C in 10 mL of APT broth. A 1 mL portion of each grown culture was pipetted into Eppendorf tubes and boiled for 1 min. Cells were removed by centrifugation at 10 000 x *g* for 10 min at 4°C. The supernatant fluid was collected and stored at 4°C until used for analysis within 1 day.

2.2.3. *Purification of enterocin B.*

Enterocin B was purified by a modified method of that described by Franz et al. (1999). *E. faecium* BFE 900 was grown for 18 h in 1 L of APT broth supplemented with 3% glucose (v/v). The culture was heated for 30 min at 75°C, and cells were removed by centrifugation (8 000 × g; 30 min). The supernatant was collected and loaded onto an Amberlite XAD-8 column (BDH Chemical Ltd., Poole, UK; 150 mm × 75 mm). The column was washed with 1 L of 0.1% trifluoroacetic acid (TFA), followed by 750 mL of 30% ethanol in 0.1% TFA. The active fraction, as determined by a spot-on-lawn assay, was eluted with 60% ethanol in 0.1% TFA, and concentrated to approximately 75 mL by rotary evaporation. The concentrated fraction was adjusted to pH 4.5 with 20 mM sodium acetate buffer (pH 5). The fraction was loaded onto a SP Sepharose Fast Flow cation exchange column (Pharmacia Biotech, Baie D'Urfe, PQ, Canada; 110 mm × 13 mm) and the column was sequentially washed with 100 mL of sodium acetate buffer, 60 ml of 100 mM sodium chloride in sodium acetate buffer, and 60 ml of 500 mM sodium chloride in sodium acetate buffer, which eluted the bacteriocin. The active fraction was loaded onto a Sep Pak C18 reverse-phase column (Waters Ltd., Mississauga, ON, Canada). The column was washed with 10 mL of Milli-Q water and then 10 mL of 30% ethanol. The bacteriocin was eluted with a final wash of 10 mL of 95% ethanol. The active fraction was freeze-dried overnight and resuspended in 0.1% TFA. At each step during the purification, aliquots of the active fractions of the supernatant, XAD-8 column, cation-exchange column, and resuspended freeze-dried protein fractions were collected and stored at 4°C for further analysis.

2.2.4. *Bacteriocin activity assay.*

The culture supernatants were assayed for activity against *C. divergens* LV 13 using the spot-on-lawn technique with APT agar (Ahn and Stiles, 1990). The plates were incubated for 18 h at 30°C. Fractions from the enterocin B purification were assayed by the same method using Lactobacillus MRS agar and *L. sakei* DSM 20017 as the indicator strain. Activity was measured by taking the reciprocal of the highest dilution that exhibited a clear zone of inhibition, and was expressed as arbitrary units per milliliter (AU/mL).

2.2.5. *MALDI-TOF MS.*

All mass spectra were acquired on a linear MALDI-TOF MS equipped with pulsed ion extraction technology (Bruker Proflex™ III, Billerica, MA) with a 125 cm flight tube. All spectra were acquired in positive ion linear mode with a nitrogen laser (λ = 337 nm) for desorption/ionization of the samples and an acceleration voltage of 20 kV. The spectra are representative of 60 consecutive laser shots. Angiotensin II (MH^+ = 1046.542; Sigma Chemical Co., St. Louis, MO) and bovine insulin (MH^+ = 5734.557; Sigma) were used as the calibrants for external mass calibration. The use of synthetic membranes and washing the probe with water were examined as methods of removing sample contaminants to achieve effective MALDI-TOF MS analysis. Polyethylene membranes (Fisher Scientific, Fair Lawn, New Jersey) were prepared by the method described by Worral et al. (1998). The membrane was saturated with methanol, air-dried, and fixed to the stainless steel probe with double-sided tape. Culture supernatant (0.5 µL) was spotted onto the membrane and allowed to dry. The membrane was washed 3 times

with 20 µL of 70% methanol in water and air-dried between each wash. A saturated solution of sinapinic acid (0.5 µL; Sigma) was spotted onto the sample. The supernatant of *L. lactis* ATCC 11454 was used to determine the most effective washing method of the sample directly on the probe (on-target washing). Supernatant samples (0.5 µL) were placed on a stainless steel MALDI probe, and the probe was allowed to air dry. The probe was dipped into water and held static for 0, 10, 30 or 60 s. The excess water was shaken off and the probe was air-dried. When dry, 0.5 µL of a saturated solution of sinapinic acid in two-parts 0.1% TFA and one-part acetonitrile was added to the sample spot and allowed to dry before analysis.

2.3. Results.

2.3.1. *Detection of bacteriocins using bioassays.*

All supernatants and fractions tested had antibacterial activity against the appropriate indicator strains (Table 2.2). The concentration of bacteriocins in the supernatants varied from 1 600 to 6 400 AU/mL when assayed against *C. divergens* LV 13. Throughout the purification procedure for enterocin B, the relative AU/mL in each of the fractions increased.

Table 2.2. Concentration (AU/ml) of bacteriocins in prepared culture supernatants and fractions collected during purification of enterocin B.

Source	Producer Strain or Sample	Indicator Organism	AU/mL
Culture Supernatant	*L. lactis* ATCC 11454	*C. divergens* LV13	1600
	B. campestris ATCC 43754	*C. divergens* LV13	6400
	P. acidilactici PAC-1.0	*C. divergens* LV13	1600
	E. faecium CTC 492	*C. divergens* LV13	3200
	E. faecium BFE 900	*L. sakei* DSM 20017	6400
Enterocin B Purification	Supernatant	*L. sakei* DSM 20017	6400
	XAD-8 column	*L. sakei* DSM 20017	12800
	cation-exchange column	*L. sakei* DSM 20017	25600
	resuspended freeze dried protein	*L. sakei* DSM 20017	204800

2.3.2. *Detection of bacteriocins using MALDI-TOF MS.*

Attempts with membranes to adsorb bacteriocins for MALDI-TOF MS analysis were unsuccessful (data not shown). However, the on-target washing method proved to be useful for the detection of bacteriocins using MALDI-TOF MS. Figure 2.1A shows the spectrum of the prepared culture supernatant from *L. lactis* ATCC 11454, which should show evidence of nisin production. The natural contaminants in the prepared culture supernatant were present in sufficient concentration to affect the quality of the protein signal. A 10 s wash with Milli-Q water removed a portion of the contaminants (Figure 2.1B); however, a 30 s wash (Figure 2.1C) was the most effective in removing the contaminants, resulting in a peptide signal of greater intensity, better resolution and less background noise. The 60 s wash (Figure 2.1D) was also effective in removing the contaminants, but it appeared to degrade the sample signal. Despite poor signal intensity and resolution for some of the samples in Figure 2.1, the m/z ratios for all samples were similar to the mass expected for nisin (MH^+ = 3 354 ± 0.1%) with signals ranging from 3355 to 3359 Da. Similar results were obtained when enterocin B was washed for the

same times (data not shown). The 30 s wash was used as the method for sample preparation when attempting to detect other bacteriocins using MALDI-TOF MS.

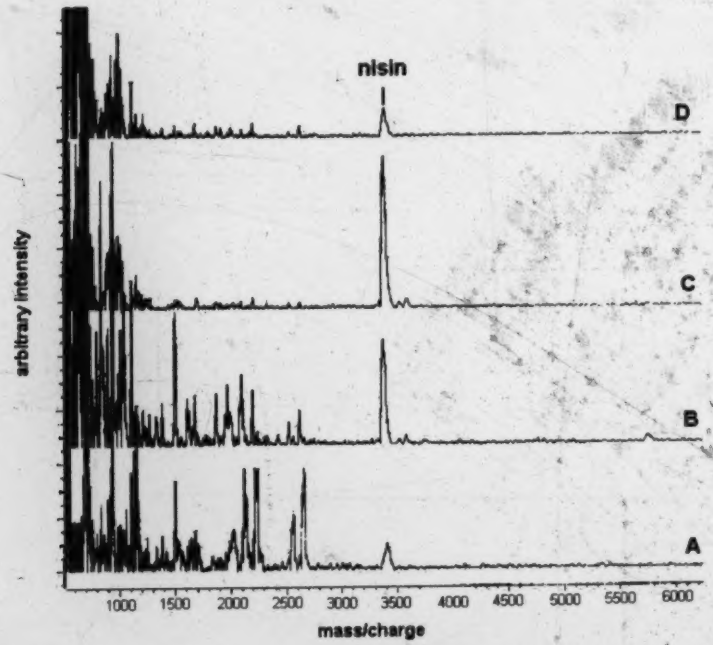

Figure 2.1. Comparison of mass spectra obtained from a crude bacteriocin preparation of nisin with either A) no water rinse; B) a 10 sec water rinse; C) a 30 second water rinse; or D) a 60 sec water rinse.

MALDI-TOF MS also detected brochocins A and B, pediocin PA-1, enterocins A and B in cell-free supernatants of the producer organisms. Figure 2.2A shows the spectra obtained with the culture supernatant of *B. campestris* ATCC 43754, which produces brochocin A (5 242 Da) and brochocin B (3 943 Da) (McCormick et al., 1998). The peak in the 2 920 Da range has not been identified. Figure 2.2B shows the MALDI-TOF MS spectra obtained from the supernatant of *P. acidilactici* PAC-1.0 that produces pediocin

PA-1 (4 629 Da) (Henderson et al., 1992). Figures 2.2C and 2.2D are the mass spectra obtained with the supernatants of *E. faecium* CTC 492 and BFE 900, respectively. *E. faecium* CTC 492 produces enterocin A, a 47 amino acid bacteriocin with a molecular mass of 4 829 Da (Aymerich et al., 1996). The MALDI mass spectra confirms that *E. faecium* CTC 492 produced enterocin A, but also shows another peptide at approximately 5 479 Da which corresponds to the mass of enterocin B (5 463 Da) (Franz et al., 1999; Nilsen et al., 1998). *E. faecium* CTC 492 is also producing another uncharacterized substance with a molecular weight of approximately 5 800 Da. The peak at approximately 5 479 Da in Figure 2.2D confirms that enterocin B is produced by *E. faecium* BFE 900, which has also been reported to produce enterocin A (Franz et al., 1999).

MALDI-TOF MS was also effective in determining the presence of bacteriocins in the active fractions obtained during the purification of enterocin B (Figure 2.3). Figure 2.3A is the MALDI spectrum for the cell-free supernatant of *E. faecium* BFE 900 with a small peak at the appropriate mass range for enterocin B. The MALDI-TOF MS spectrum for the fraction collected after purification on the XAD-8 column (Figure 2.3B) also confirms the presence of enterocin B. Figure 2.3C shows that enterocins A and B are present in the fraction eluted from the cation exchange column. Figure 2.3C is the MALDI-TOF MS spectrum of the sample after resuspension of the freeze-dried bacteriocin. Throughout the purification, with increasing bacteriocin concentration, the arbitrary intensity of the peaks increased.

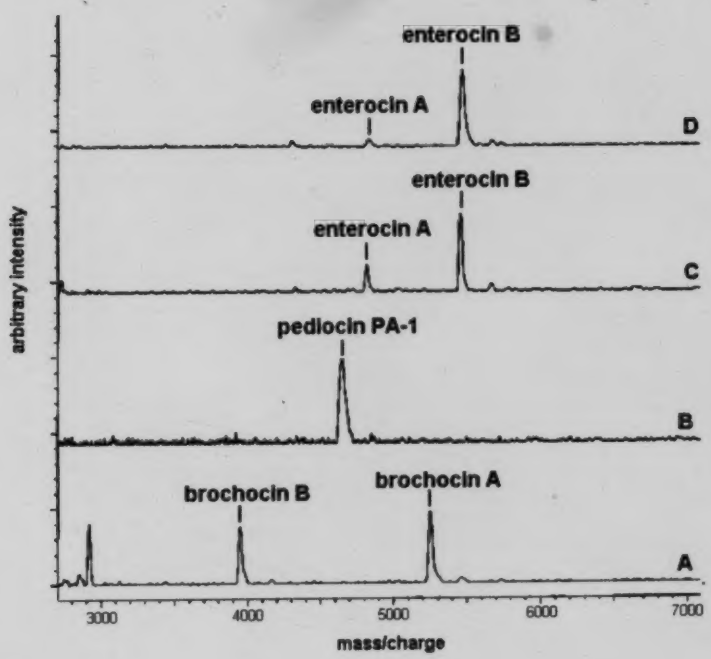

Figure 2.2. Mass spectra obtained from crude bacteriocin preparations from A*) B. campestris* ATCC 43754; B) *P. acidilactici* PAC-1.0; C) *E. faecium* CTC 492; and D) *E. faecium* BFE 900.

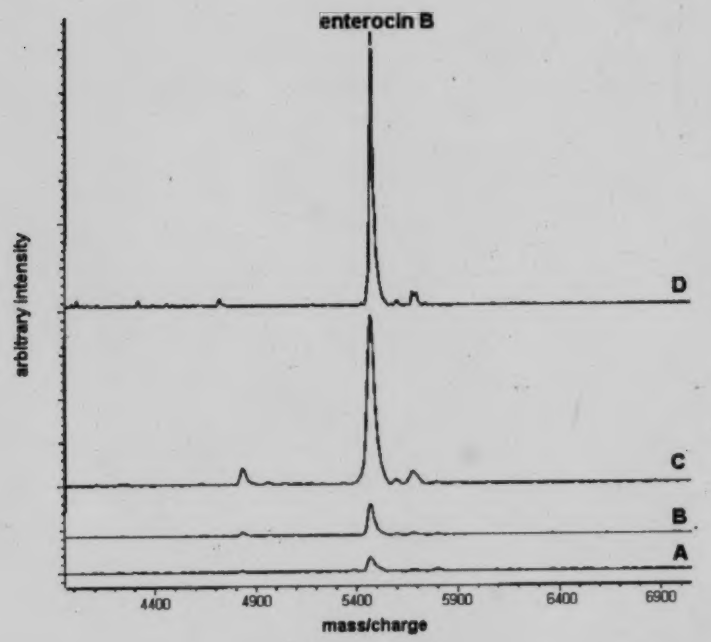

Figure 2.3. Mass spectra obtained from fractions of an enterocin B purification A) supernatant; B) after separation on XAD-8 column; C) after separation on cation-exchange column; and D) resuspended freeze-dried protein.

2.4. Discussion.

MALDI-TOF MS is the first reported mass spectrometric technique to be used to detect bacteriocins in the cell-free supernatants of a bacterial culture. This is largely due to the ability to purify samples on-target. In our studies, a sterile water wash was chosen as the method for removing contaminants. Other researchers have suggested the use of polyethylene or polypropylene membranes for use as activated membranes to bind peptides or proteins (Blackledge and Alexander, 1995a.b; Worrall et al., 1998). However, Joosten and Nũnez (1995) reported that Tween 80 prevents adsorption of the bacteriocins nisin and enterocin on polypropylene surfaces. This may explain the loss of peptide signal found when we studied the use of polyethylene membranes with various rinses as a sample surface for MALDI-TOF MS analysis.

The presence of contaminants in the culture supernatant greatly suppresses the signal of the peptide. Blackledge and Alexander (1995b) suggested that the contaminants prevent effective crystallization of the matrix and are desorbed with more efficiency than the peptide. This, in turn, suppresses the peptide signal. Amado et al. (1997) indicated that the loss of protein signal may be explained by partial precipitation of surfactant-protein ionic pairs during sample preparation. They also suggested that the signal degradation found with high concentrations of surfactants may be the result of surfactants coating the matrix crystals, thus diminishing energy transfer and desorption/ionization efficiency. The results presented in this study show that using a 30 second water rinse in sample preparation removes the majority of the contaminants, resulting in a better S/N ratio, better peak resolution and better signal intensity of the sample peak. Although a 30

s wash is optimum in this study, other time lengths may be effective depending on the amount of interfering contaminants in the sample.

There are slight discrepancies in the molecular masses given by MALDI-TOF MS compared with the reported masses of the bacteriocins in the literature (Table 2.1). This could be the result of many factors. For example, the mass of enterocin B determined by MALDI-TOF MS (5 479 Da) was 16 Da greater than that reported in the literature. This difference is likely due to oxidation of the peptide. Wang et al. (1998) attributed mass discrepancy to difficulty in accurately determining the peak centroid due to the low resolving capabilities of MALDI-TOF MS, as well as the use of external versus internal calibration. External calibration is the method of choice when speed and sample consumption rather than mass accuracy are of interest. A lower mass accuracy is obtained with external calibration because slight changes in laser power and sample preparation may cause differences in the desorption/ionization process. However, an advantage of MALDI-TOF MS is that the results are the average of many individual laser pulses (Takach et al., 1997). Therefore, the combination of a large number of ions and good calibration should alleviate concern regarding the accuracy of the mass. In this study, it was shown that MALDI-TOF MS should not be relied upon for an accurate molecular weight; however it is capable of providing reproducible spectra for the detection of bacteriocins within the expected mass range.

It has also been shown that MALDI-TOF MS can be used to identify components of various samples throughout the bacteriocin purification process and has potential for future use in the detection of bacteriocins in genetic and food experiments. Of particular interest is the potential of MALDI-TOF MS as a quantification tool. Bouksaïm et al.

(1998) reported that to use bacteriocins as food preservatives, it is important to understand the relationship between activity and exact quantity or real concentration of bacteriocin in a food system. However, in order to quantify bacteriocins using MALDI-TOF MS, a pure sample would be needed. The method proposed in this paper for detection would not be very reproducible for quantification given the evidence that spectral changes occur with washing (Figure 2.1). Purification methods for other bacteriocins are being developed and these will be examined by MALDI-TOF MS.

Analysis by MALDI-TOF MS is relatively new and more work is needed in obtaining higher resolution spectra, better sensitivity, better sample preparation and faster data analysis (Amado et al., 1997; Costello, 1997; Takach et al., 1997; Worrall et al., 1998). The results presented here show that MALDI-TOF MS is a rapid and sensitive detection method for bacteriocins. Its ability to generate mass spectra from the supernatant makes it particularly attractive for use in industry and commercial application. Another major advantage of MALDI-TOF MS is its potential to screen supernatant and purification samples for bacteriocin production. This process takes minimal time (minutes) compared with the overnight incubation of traditional bioassays. Further research will involve using MALDI-TOF MS to detect bacteriocins in multi-component food systems and to examine the interaction between the bacteriocins and food components.

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CHAPTER 3*

INACTIVATION OF NISIN BY GLUTATHIONE IN FRESH MEAT

3.1. Introduction.

Nisin belongs to a class of bacteriocins known as lantibiotics which are small peptides defined by their antibacterial activity and the presence of lanthionines and unusual amino acids (Nes and Tagg, 1996; van De Ven and Jung, 1996). The structures of nisin and its unusual amino acids are shown in Figure 1.1. Nisin exhibits antibacterial activity against many gram-positive bacteria; however, it has no effect against gram-negative bacteria, yeast or molds (Delves-Broughton et al., 1996). The phospholipid component of the cytoplasmic membrane of vegetative cells is thought to be the major target for nisin activity (Abee et al., 1995); whereas nisin acts on the sulfhydryl membrane groups for inactivation of germinated spores (Liu and Hansen, 1990; Morris et al., 1984). Nisin has been approved as a food additive in over 46 countries for use in processed cheeses, dairy products and canned foods (Delves-Broughton et al., 1996). However, the use of nisin in meats is limited because various meat components interfere with its activity (Henning et al., 1986b).

Several methods have been examined as potential methods for determination of the concentration of nisin in food products. Researchers typically rely on extraction and bioassays for the detection of bacteriocins in meats (Leistner and Gorris, 1995; McMullen

* A version of this chapter entitled **Inactivation of nisin by glutathione in fresh meat** was published in 1999 in *Journal of Food Science* 64(5):759-762 by Natisha L. Rose, Peter Sporns, Michael E. Stiles and Lynn M. McMullen.

and Stiles, 1996). Recently, Bouksaïm et al. (1998) used enhanced chemiluminescence for the detection of nisin in milk and whey. Each method is effective; however, both are time-consuming and neither allows for the study of the interaction of the bacteriocin with the food components.

MALDI-TOF MS has been shown to be an effective method for the detection of bacteriocins in the culture supernatant of producer organisms, and it has potential as a method for determining the fate of bacteriocins in foods (Rose et al., 1999). This study used MALDI-TOF MS to study the fate of nisin meat.

3.2. Materials and Methods.

3.2.1. *Bacterial strains and media.*

Carnobacterium divergens NCFB 2855 was used as a sensitive indicator for detection of nisin activity. A frozen stock culture was maintained at –70°C in Bacto APT broth (Difco Laboratories, Detroit, MI) supplemented with 20% glycerol (v/v). Prior to experimental use, *C. divergens* NCFB 2855 was subcultured twice and grown overnight in APT broth. Solid agar medium was prepared by adding 1.5% (w/v) granulated agar (Difco) to APT broth. Soft APT agar was prepared by adding 0.75% agar (w/v).

3.2.2. *Nisin solutions and meat preparation.*

Pure nisin (Aplin & Barrett Ltd., Langford, England) was stored at 4°C in a desiccator. Working solutions were prepared by dissolving 50, 125, and 250 µg of nisin in 1 mL of 0.02 N HCl (pH 2). A solution of 0.02 N HCl was used as a control.

Fresh eye-of-round beef was obtained from a local supermarket. Pork was obtained from a federally-inspected processing plant, stored at -30°C and thawed at room temperature before use in experiments. Meat samples (1.0 × 1.0 × 0.5 cm) were soaked in 1 mL of control and nisin solutions for 1 h at 4°C. Cooked beef samples were prepared by heating the raw beef samples for 5 min in a boiling water bath and allowing the meat to cool to 4°C before use in the experiments (within 1 h after cooking). Raw beef samples were soaked in each nisin solution and samples of raw pork and cooked beef were soaked in the solution containing 250 μg nisin/mL. After soaking in the nisin solution, the meat samples were individually vacuum-packaged and stored overnight at 4°C. After the meat was removed from the nisin solutions, the residual solutions were stored at 4°C for nisin activity and MALDI-TOF MS analysis.

To prepare raw and cooked beef juices, fresh eye-of-round beef was pummeled in a Colworth 400 Stomacher (Seward and Co., London, England) for 1 min and the resulting juice was collected. A portion of the juice was heated in a boiling water bath for 5 min. For both raw and cooked beef juice, one part of beef juice was combined with one part of nisin (250 μg/mL) solution, held at 4°C overnight and centrifuged at 8160 × *g* for 2 min before analysis.

3.2.3. *Nisin extraction and activity assay.*

After overnight storage, 250 μL of 0.02 N HCl was added to each meat sample. The samples were heated at 100°C for 1 min to inactivate endogenous enzymes, macerated by hand, and blended in a Colworth 400 Stomacher for 1 min. The suspension

was centrifuged at 8160 x *g* for 2 min. The extracts were analyzed by MALDI-TOF MS and assayed for antibacterial activity against *C. divergens* NCFB 2855 using the spot-on-lawn technique with APT agar (Ahn and Stiles, 1990). The original nisin solutions and the 0.02N HCl solution were also assayed. The plates were incubated for 18 h at 30°C in an anaerobic jar flushed with a gas mixture of 10% CO_2 and 90% N_2. Activity was measured by taking the reciprocal of the highest dilution that gave a clear zone of inhibition and was expressed as arbitrary units per milliliter (AU/mL).

3.2.4. *MALDI-TOF MS.*

All mass spectra were acquired on a linear MALDI-TOF MS equipped with pulsed ion extraction technology (Bruker Proflex™ III, Billerica, MA) and a 125 cm flight tube, in a positive ion linear mode with a nitrogen laser (λ=337 nm) for desorption/ionization of the samples and an acceleration voltage of 20 kV. The spectra are representative of 100 consecutive laser shots. Angiotensin II (MH^+ = 1 046.542) and bovine insulin (MH^+ = 5 734.557), obtained from Sigma Chemical Co. (St. Louis, MO), were used as calibrants for external mass calibration.

Samples were prepared for MALDI-TOF MS analysis by the method described by Rose et al. (1999; Chapter 2, this thesis). Samples for analysis included initial and residual nisin solutions, extracts of raw and cooked meat soaked in nisin, and the raw and cooked juice with nisin added. The supernatant (0.5 µL) of meat extract and juice samples was placed on a stainless steel MALDI probe and allowed to air dry. The probe was dipped into Milli-Q water and held static for 30 s to remove water-soluble contaminants from the sample. The excess water was shaken off the probe, and the sample was air-

dried. When dry, 0.5 µL saturated solution of sinapinic acid (Sigma) in a solution containing two parts of 0.1% TFA and one part of acetonitrile was added to the sample spot. The MALDI-TOF MS samples for the initial nisin solutions were prepared without the washing step because of the pure nature of the samples.

3.3. Results.

3.3.1. *Nisin assays.*

Prior to soaking the meat, the control (0.02 N HCl) and nisin solutions were assayed by the spot-on-lawn method (Ahn and Stiles, 1990) for the number of arbitrary units of bacteriocin using *C. divergens* NCFB 2855 as the indicator strain (Table 3.1).

Table 3.1. Activity (AU/mL) of control and nisin solutions.

Nisin concentration (µg/ml)	Activity units (AU/ml)
0[a]	0
50	1600
125	3200
250	6400

[a] 0.02 N HCl

Each solution was also analyzed by MALDI-TOF MS to determine the presence of nisin. The mass spectra of the control solution and the solution containing 250 µg/mL nisin in 0.02 N HCl are shown in Figure 3.1A. Spectra similar to that shown in Figure 3.1B were obtained for the solutions containing 50 and 125 µg/mL nisin.

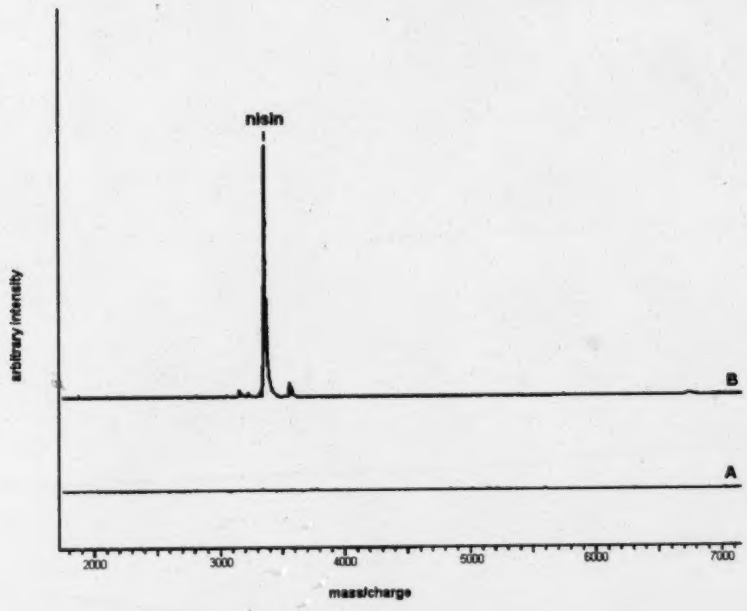

Figure 3.1. Mass spectra of (A) control solution of 0.02 N HCl, and (B) 250 µg/mL solution of nisin in 0.02 N HCl. Average mass of nisin solution was 3365.77 ± 1.76 Da (n=4).

Results for bacteriocin activity in the supernatants of residual nisin solutions, meat juices and meat extracts are given in Table 3.2. All residual nisin solutions contained some antibacterial activity; however, the activity was approximately half that of the initial nisin solutions. Antibacterial activity was detected in the extract obtained from the cooked meat sample; however, no activity was detected in any of the extracts from the raw meat samples. Antibacterial activity was found in the juice obtained from the raw and cooked meat, although the juice from the cooked meat had four times the activity of the juice from the raw meat.

Table 3.2. Concentration of nisin recovered from supernatant of residual nisin solutions, meat juice and meat extracts. Activity was determined against *C. divergens* NCFB 2855.

Meat	Preparation	Nisin concentration (μg/mL)	Activity (AU/mL) Raw	Cooked
Beef	Residual nisin solution[a]	50	800	n.d.
		125	1600	n.d.
		250	3200	3200
	Meat Extract[a]	50	0	n.d.
		125	0	n.d.
		250	0	3200
	Meat Juice[a]	250	400	1600
Pork	Meat Extract[a]	250	0	n.d.

[a]n=4
n.d. not determined

3.3.2. *MALDI-TOF MS analysis.*

MALDI-TOF MS analysis was performed on all residual nisin solutions and on the supernatant from the juice and extracts of the meat samples. Figure 3.2A and 3.2B show the mass spectra of the residual nisin solutions (250 μg/mL) of cooked and raw beef. A single peak corresponding to the mass of nisin was observed for the residual solution of the cooked meat (Figure 3.2A); whereas, the nisin signal and an additional peak approximately 307 Da greater than the nisin peak was detected in the supernatant of the fresh meat extract (Figure 3.2B). Similar results were obtained for the supernatants of the raw and cooked meat juice samples (Figure 3.2C and 3.2D). Again, a second peak 307 Da greater that that of nisin was detected only in the raw meat juice.

The mass spectra of the cooked beef extract (Figure 3.3A) shows a typical nisin ion peak; whereas no comparable signal is observed in either the raw pork or raw beef extracts (Figure 3.3B and 3.3C, respectively). However, a signal approximately 307 Da greater than the mass of nisin was observed for the raw pork and raw beef extracts.

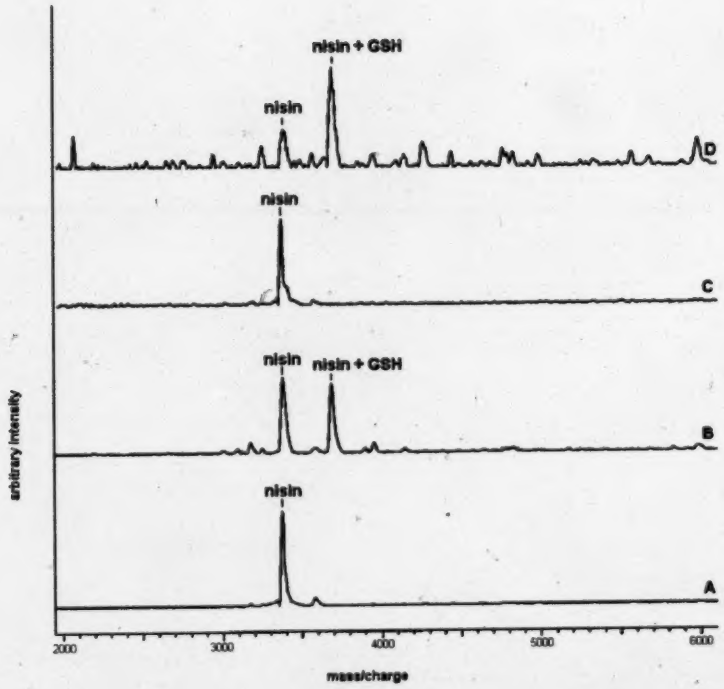

Figure 3.2. Mass spectra of the residual nisin solution (250 μg/mL) of (A) cooked beef, (B) raw beef, (C) cooked meat juice, and (D) raw meat juice. Average mass for (A) was 3365.51 ± 0.5 Da (n=4), and the average measured mass difference for the two peaks in (B) was 307.62 ± (0.2) Da (n=4). Average mass for (C) was 3366.35 ± 1.8 Da (n=4), and average mass difference for the two peaks in (D) was 307.57 ± 0.2 Da (n=4).

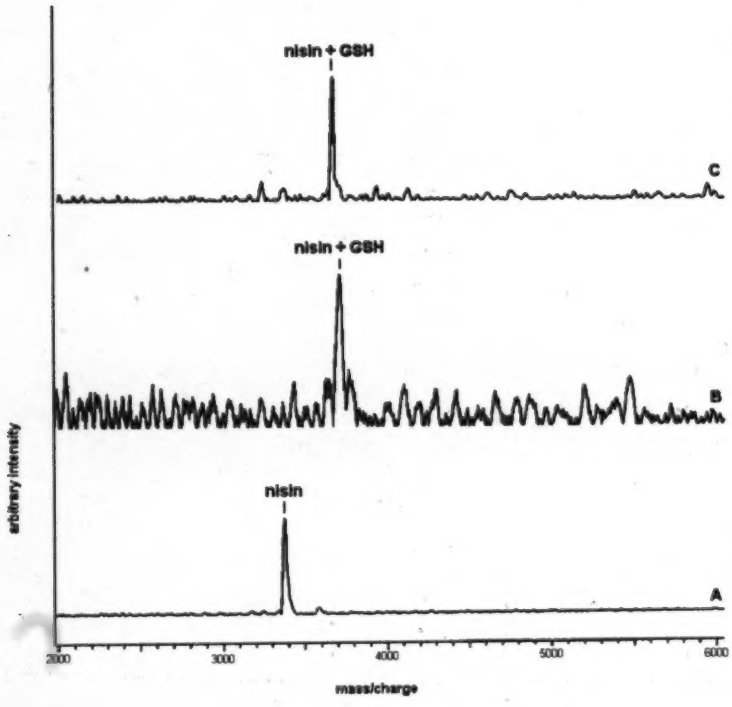

Figure 3.3. Mass spectra of meat extract from (A) cooked beef, (B) raw pork, and (C) raw beef. All samples were soaked in a nisin (250 μg/mL) solution. Averaged masses were (A) 3365.56 ± 1.1 Da (n=4), (B) 3673.78 ± 1.12 Da (n=4), and (C) 3672.74 ± 1.8 Da (n=4).

3.4. Discussion.

Despite its reported activity against the outgrowth of spores and gram-positive foodborne pathogens, the use of nisin in meat products has been unsuccessful, unless used in high concentrations (Bell and Lacy, 1986; Chung et al., 1989; Mahadeo and Tatini, 1994; Rayman et al., 1983; Scott and Taylor, 1981; Stevens et al., 1991). There are many theories for why nisin is inactive in meat; however, there is no scientific evidence to substantiate the theories. Its ineffectiveness in meat products has been

attributed to reactions with meat components and surfaces, poor solubility, sensitivity to food enzymes, high bacterial loads, interaction with phospholipids and poor distribution throughout the meat product (Bell and Lacy, 1986; Delves-Broughton et al., 1996; Henning et al., 1986a; Scott and Taylor, 1981; Stringer et al., 1995). These problems are reportedly due primarily to the hydrophobic nature of nisin and its instability at neutral pH (Delves-Broughton et al., 1996; Henning et al., 1986b; Scott and Taylor, 1981; Stringer et al., 1995).

With the use of MALDI-TOF MS and activity assays, we were able to determine the fate of nisin on raw meat. Extracts of raw meat samples that had been soaked in a nisin solution were lacking in nisin activity; whereas, those from cooked meat retained activity. The results also showed that the mass of nisin increased by approximately 307 Da when it was applied to fresh meat and stored. In contrast, when nisin was applied to cooked meat, there was no shift in mass. These results indicated that nisin reacted with a component of fresh meat and that the reaction was likely enzyme driven because it did not occur in cooked meat. The mass difference of 307 Da was suspected to be due to a reaction between nisin and glutathione (MW = 307.33). Reactions with glutathione are typically enzyme-mediated; therefore, the extract of cooked meat was analyzed to determine if this reaction would occur once the enzymes were inactivated.

Glutathione (N-(N-L-γ-glutamyl-L-cysteinyl)glycine) is a major low molecular weight (307 Da) thiol compound found in both prokaryotic and eukaryotic cells. Its structure is shown in Figure 3.4. GSH is widespread in nature and is found in both plant and animal tissues.

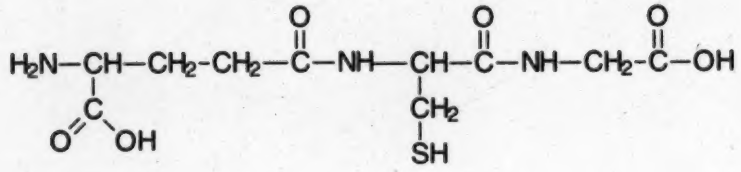

Figure 3.4. The structure of glutathione.

Wierzbicka et al. (1989) examined the amount of GSH in foods consumed by humans. They found that the concentration of GSH in beef, chicken and pork was generally high with 156 to 627 nmol/g wet weight. The concentration of GSH is less in raw fish (21 nmol/g wet weight), and there is essentially no GSH in dairy products. GSH functions in the body to detoxify carcinogenic electrophiles and to protect cells against oxidative damage, and GSH reacts with electrophiles to yield GSH S-conjugates (Wierzbicka et al., 1989). These reactions are partially mediated by glutathione S-transferase and by glutathione peroxidase. Lee et al. (1996) found that glutathione peroxidase is present in high amounts in fresh turkey thigh muscle (0.73 ± 0.04 U/g), and it is recovered in very low amounts (0.03 ± 0.01 U/g) after cooking. The amount of glutathione in poultry meat is unaffected by heat. Williamson and Ball (1988) postulated that glutathione S-transferase may protect against oxidative damage in meat and they isolated the enzyme from fresh pork to study its properties. Their results showed that the enzyme was most stable and active at pH 5.5 to 6.0, which was consistent with the pH of fresh meat products.

The enzyme responsible for driving the reaction between nisin and glutathione is glutathione S-transferase. Nisin exhibits some features common to known substrates of

glutathione S-transferase, including its hydrophobicity and the fact that it contains an electrophilic carbon atom (Sipes and Gandolf, 1991). Glutathione S-transferase catalyzes the reaction of glutathione with compounds containing electrophilic carbon atoms, forming a thio-ether bond between the carbon atom and the sulfhydryl group of glutathione (Sipes and Gandolf, 1991). This general type of reaction is shown in Figure 3.5. In the presence of an electrophile, mercaptans can add to a double bond; this addition can be done with GSH and glutathione S-transferase.

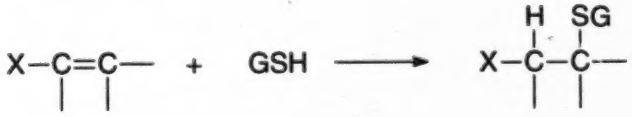

Figure 3.5. A general reaction catalyzed by glutathione S-transferase. X represents an electron-withdrawing group.

Several reports in the literature have demonstrated that nisin readily reacts with free sulfhydryl groups. Nisin has been shown to be an effective inhibitor of *B. cereus* and *C. botulinum* spore outgrowth by inactivating the membrane sulfhydryl groups present in the newly germinated spores (Liu and Hansen, 1990; Morris et al., 1984; Scott and Taylor, 1981; Somers and Taylor, 1981). The sulfhydryl groups are involved in many enzymatic reactions of the cell and, therefore, serve as a potential site of inhibition by sulfhydryl agents. A good sulfhydryl agent has been defined as a compound of moderate reactivity in a relatively bulky, charged and nontransportable molecule (Morris et al., 1984). Nisin meets the criteria of a good sulfhydryl agent because it is a 34-residue peptide that is relatively bulky, and its activity is associated with the unusual dehydro

residues that could act as electrophilic Michael acceptors and readily react with mercaptans and other good nucleophiles (Liu and Hansen, 1990; Morris et al., 1984). Furthermore, it has been reported that the activity of nisin is directly related to the presence of dehydroalanine (Gross and Morell, 1967; Liu and Hansen, 1990). The reaction of nisin with mercaptans results in the complete loss of intact DHA residue at position 5 (Liu and Hansen, 1990). Gross and Morrell (Gross and Morell, 1967) suggested that metabolically important compounds, such as sulfhydryl-containing enzymes, glutathione (GSH) or co-enzyme A, may be intercepted by nisin. Based on the mass shift of 307 Da, it is likely that nisin is reacting enzymatically with GSH and that this reaction would result in the loss of nisin activity. Moreover, the lack of reaction in cooked meat was not due to depleted GSH, because addition of fresh GSH to cooked meat juice did not result in a nisin peak with the 307 Da mass increase (unpublished data).

Our studies have illustrated the utility of MALDI-TOF MS in determining the fate of peptides, such as bacteriocins, in complex food systems such as meat. The data presented in this study supports the hypothesis that nisin is inactivated in raw meat due to a specific reaction with a compound in raw meat. Due to the noted mass increase of nisin, that compound is almost certainly glutathione. From the inhibition studies, it is clear that nisin is inactivated when bound to glutathione. Glutathione is not found in dairy products, which would explain why nisin is effective as an antibacterial agent in dairy products. Future work in this area involves determining the rate of reaction between glutathione and nisin, inhibition studies on glutathione S-transferase and the potential for the use of nisin in a cooked meat product.

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CHAPTER 4*

NISIN: A NOVEL SUBSTRATE FOR GLUTATHIONE-S-TRANSFERASE ISOLATED FROM FRESH BEEF

4.1. Introduction.

Nisin is a 34-amino acid peptide belonging to a class of bacteriocins called lantibiotics. Lantibiotics are characterized by their antibacterial activity and presence of lanthionines and unusual amino acids (Nes and Tagg, 1996). Nisin belongs to the subgroup type A lantibiotics which are elongated, flexible molecules with a net positive charge that exhibit antibacterial activity by means of pore formation (Guder et al., 2000). The structure of nisin and its unusual amino acids are shown in Figure 1.1. Nisin contains dehydro residues [dehydroalanine (DHA) and dehydrobutyrine (DHB)] and thioether crosslinkages [lanthionine and β-methyllanthionine] that arise from posttranslational modification of serine, threonine and cysteine (Kuipers et al., 1996; van de Ven and Jung, 1996). The dehydro residues contain electrophilic carbon atoms that act as Michael receptors and readily react with mercaptans and other effective nucleophiles (Morris et al., 1984; Liu and Hansen, 1992).

Nisin exhibits antibacterial activity against many gram-positive bacteria, including the spores of *Bacillus* and *Clostridium* spp.; however, it has no activity against gram-negative bacteria, yeast or molds (Delves-Broughton et al., 1996). The mechanism of

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action of nisin on bacteria has not been fully elucidated; however, the phospholipid component of the cytoplasmic membrane, particularly the lipid II component, of vegetative cells is thought to be the major target for nisin activity (Breukink et al., 1999). For the inactivation of bacterial spores, it is generally accepted that nisin acts on the sulfhydryl membrane groups for inactivation of germinated spores (Morris et al., 1984). Nisin has been approved as a food additive in over 46 countries for use in processed cheeses, dairy products and canned foods (Delves-Broughton et al., 1996). However, the use of nisin in meat products has been unsuccessful, unless used in high concentrations (Bell and Lacy, 1986; Chung et al., 1989; Mahadeo and Tatini, 1994; Rayman et al., 1983; Scott and Taylor, 1981; Stevens et al., 1991). Several theories have been proposed for the lack of nisin activity in meat; however, scientific evidence to substantiate any of these theories was lacking. Nisin's ineffectiveness in meat products has been attributed to reactions with meat components and surfaces, poor solubility, sensitivity to food enzymes, high bacterial loads, interaction with phospholipids and poor distribution throughout the meat product (Bell and Lacy, 1986; Cutter and Siragusa, 1996; Delves-Broughton et al., 1996; Henning et al., 1986; Scott and Taylor, 1981; Stringer et al., 1995). These problems were reportedly because of the hydrophobic nature of nisin and its instability at neutral pH (Delves-Broughton et al., 1996; Stringer et al., 1995).

Our laboratory recently hypothesized that nisin is inactivated in fresh meat by an enzymatic reaction with glutathione (GSH; Rose et al., 1999; Chapter 3, this thesis). Glutathione (N-(N-L-γ-glutamyl-L-cysteinyl)glycine) is a major low molecular weight (307 Da) thiol compound found in many eukaryotic and prokaryotic cells. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS),

we showed a mass shift of ~307 Da for nisin occurred after it was recovered from the surface of fresh meat that was soaked in nisin and stored overnight. A comparable mass shift did not occur when a cooked meat sample was examined. The enzyme responsible for driving the reaction between nisin and GSH is called glutathione S-transferase (GST). Nisin exhibits some features common to known substrates of GSTs, including its hydrophobicity and the fact that it contains an electrophilic carbon atom (Sipes and Gandolf, 1991).

Considering that GSH is an abundant thiol compound in animal tissues and that sulfhydryl groups are a known target of nisin, it seems likely that nisin is easily intercepted by GSH, as was suggested by Gross and Morell (1971). To examine this reaction *in vitro*, GST was isolated from fresh beef and used to catalyze the reaction between pure nisin and glutathione. The reaction was analyzed by MALDI-TO MS and antibacterial activity assays.

4.2. Materials and Methods.

4.2.1. *Bacterial strains and media.*

All bacterial strains used in this study were maintained as frozen stock cultures at -70°C in Bacto APT broth (Difco Laboratories, Detroit, MI) supplemented with 20% glycerol (v/v). Prior to experimental use, the cultures were subcultured twice and grown overnight in APT broth. Solid agar medium was prepared by adding 1.5% (w/v) granulated agar (Difco) to APT broth. Soft APT agar was prepared by adding 0.75% agar (w/v).

4.2.2. *Purification of glutathione S-transferase.*

GST was purified from fresh meat by the method reported by Williamson and Ball (1988). Briefly, fresh beef round eye of round steak (430 g), obtained from a local supermarket, was diced and washed in 0.11 M NaCl. 2 L of 0.25 M sucrose/1 mM EDTA/ 0.01 M potassium phosphate (pH 6.5; 0.1% aprotinin) was added, and the mixture was blended for one minute at maximum speed. The enzyme extract was collected after centrifugation for 1.5 h at 2 500 $\times g$ and filtered through Whatman # 1 paper to remove floating lipids. The extract was then subjected to a series of chromatography methods. First, it was passed through a DEAE Sephadex A50 column to remove non-protein A_{280} absorbing material and also to prevent subsequent blocking of S-hexylglutathione Sepharose. GST was then isolated after application to an affinity chromatography column, which was prepared as described by Mannervik and Guthenberg (1981). The eluted fraction exhibiting absorbance at 280 nm was dialyzed against 4 changes of 0.01 M potassium phosphate (pH 6.0)/1 mM EDTA over 24 h before being freeze-dried. The amount of enzyme purified was determined by the SDS Lowry method for protein determination. Purity of the enzyme was confirmed using SDS-Page gel electrophoresis and isoelectric point determination.

4.2.3. *Kinetic properties of GST.*

Enzyme activity during purification was determined by using a spectrophotometric assay described by Habig and Jakoby (1981). The formation of the conjugate of GSH (1.0 mM) and 1-chloro-2,4-nitrobenzene (CDNB; 1.0 mM) in 1.0 M potassium phosphate (pH 6.5) was measured spectrophotometrically at 340 nm. An assay

mixture without GST was performed as a control, and the rate in absence of the enzyme was subtracted to correct for the non-enzymatic reaction. A unit of enzyme was defined as the amount of enzyme required to catalyze the formation of 1 μmol of *S*-2,4-dinitrophenylglutathione per minute at 25°C using 1.0 mM concentrations of GSH and CDNB (Mannervik and Guthenberg, 1981).

4.2.4. *Stability of isolated glutathione S-transferase.*

The isolated GST was examined for pH and temperature stability. To determine the pH stability, GST (10 μg) was dissolved in 0.1 M potassium phosphate with pH values ranging from 2 to 10. For temperature stability, GST (10 μg) was dissolved in 0.1 M potassium phosphate (pH 6.5) and held at temperatures ranging from 25 to 100 °C (increasing by 5 °C increments) for 1 min and 5 min. A fraction of each GST sample (10 μL) was added to glutathione and CDNB, and the reaction was measured spectrophotometrically as described previously. The temperature or pH sample exhibiting the greatest absorbance at 340 nm was assigned 100% stability, and all others were values were assigned as a fraction of the most stable temperature and pH value.

4.2.5. *Nisin assay.*

Stock solutions of nisin (100 μg/mL) and purified glutathione S-transferase (1 mM) were dissolved in a 50 mM sodium phosphate buffer (pH 6.0). Glutathione was dissolved in the same buffer at concentrations ranging from 10 to 500 mM, and the solution was adjusted to pH 6.0. Final working conditions of the assay consisted of glutathione (5 to 250 mM), nisin (50 μg/mL) and glutathione S-transferase (1 μM). A

control reaction consisted of the same concentrations of nisin and glutathione in the absence of the enzyme. Reactions were carried out at 4°C, room temperature and 37°C. The reaction was stopped after 24 hours by adding trifluoroacetic acid to a final concentration of 0.6%. The products were analyzed by MALDI-TOF MS and for antibacterial activity.

4.2.6. *MALDI-TOF MS.*

All mass spectra were acquired on a linear MALDI-TOF MS equipped with pulsed ion extraction technology (Bruker Proflex™ III, Billerica, MA) and a 125 cm flight tube, in a positive ion linear mode with a nitrogen laser (λ=337 nm) for desorption/ionization of the samples and an acceleration voltage of 20 kV. Samples were prepared for MALDI-TOF MS analysis by the method described by Rose et al. (1999). A saturated solution of the matrix, sinapinic acid (Sigma) was prepared in a solution containing two parts of 0.1% TFA and one part of acetonitrile. The sample (10 µL) and matrix (10µL) were mixed, and 2 µL was spotted on a stainless steel probe and allowed to air dry. The spectra are representative of 50 consecutive laser shots. Bovine insulin (MH^+ = 5 734.557; MH_2^{2+} = 2 867.782), obtained from Sigma Chemical Co. (St. Louis, MO), was used as a calibrant for external mass calibration.

4.2.7. *Antibacterial activity assay.*

To determine the extent of antibacterial activity loss, each sample was assayed against *Lactobacillus sakei* DSM 20017 and *Carnobacterium divergens* NCFB 2855 using the spot-on-lawn technique with APT agar (Ahn and Stiles, 1990). Those samples

exhibiting activity were assayed for total activity by doubling dilutions. Total activity was determined by taking the reciprocal of the highest dilution that gave a clear zone of inhibition and was expressed as arbitrary units per milliliter (AU/mL).

4.2.8. *HPLC Chromatography.*

Attempts were made to separate the reactant products from nisin using high-performance liquid chromatography (HPLC). Analysis was carried out on a Varian 5000 liquid chromatograph system with a Waters 486 tunable absorbance detector. A Phenomenex® Jupiter C_4 column (250 × 4.60 mm packed with 5 µm beads; Torrence, CA) was used. Water and acetonitrile in 0.1% TFA were used as solvents. An elution gradient (1 mL/min) of 0 to 100% acetonitrile over 45 minutes was used. Fractions exhibiting absorbance at 220 nm were pooled and dialyzed against 4 changes of water over 24 h. The fractions were freeze-dried and resuspended in 50 mM phosphate buffer (pH 6.0) for MALDI-TOF MS and antibacterial activity analyses.

4.3. Results.

4.3.1. *Purification of GST.*

Glutathione S-transferase (GST) was successfully extracted and isolated from an extract of fresh ground beef using a series of chromatography methods. The results of the purification are shown in Table 4.1.

Table 4.1. Purification of glutathione S-transferase from fresh beef.

Fraction	Volume (mL)	Total protein[a] (mg)	Specific activity (units/mg)	Total activity (units)	Yield (%)
Supernatant	1940	35 520	0.045	1596.4	100
DEAE Sephadex A50	1800	23 950	0.063	1508.8	94.4
Sepharose 6B	73	78.6	4.3	337.6	21.12
Dialysis	60	44.7	13.51	603.90	37.78

[a] Total protein was determined by the SDS Lowry method for protein determination.

A final specific activity of 13.51 units/mg and a 300.2 -fold purification indicates that approximately 0.3% of the total soluble protein in bovine muscle is GST. This value is greater than the 0.06% reported in pig muscle; however closer to that of other animal muscle (Williamson and Ball, 1988). The isolated enzyme is also in the appropriate range for subunit size (~20 000 to 25 000 Da) and isoelectric point (~ 7 to 7.5) for known GSTs. Both SDS-page gel electrophoresis and isoelectric point determination showed one band at ~20 195 Da (Figure 4.1) and 7.33 (Figure 4.2) respectively.

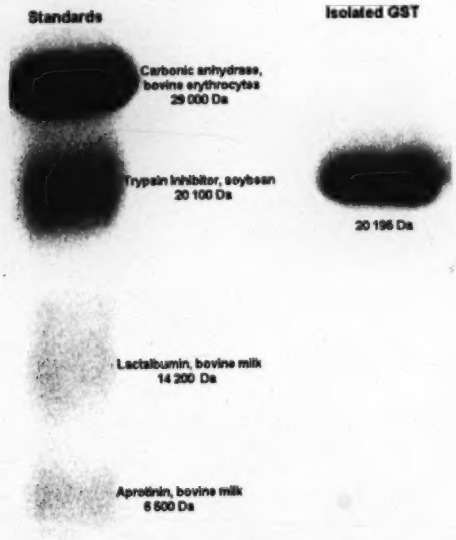
Standards
Isolated GST
Carbonic anhydrase, bovine erythrocytes 29 000 Da
Trypsin inhibitor, soybean 20 100 Da
20 196 Da
Lactalbumin, bovine milk 14 200 Da
Aprotinin, bovine milk 6 500 Da

Figure 4.1. SDS-Page gel electrophoresis of glutathione S-transferase isolated from bovine muscle.

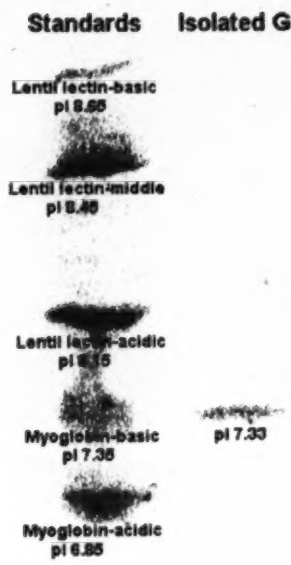

Figure 4.2. Isoelectric focusing of glutathione S-transferase isolated from bovine muscle.

4.3.2. *Stability of glutathione S-transferase.*

To elucidate the effectiveness of GST in meat, it is important to determine its stability under different conditions. The results of the pH and temperature stability are shown in Figure 4.3. At room temperature, the enzyme is most stable at pH 6.5, but it is stable between pH 5.5 and 9.5. At 4°C, the activity of GST also peaks at pH 6.5; however, its activity remains stable up to pH 9.0. GST starts to lose activity after being held at 35° for either 1 or 5 minutes. At both times, activity is completely lost at 70°C.

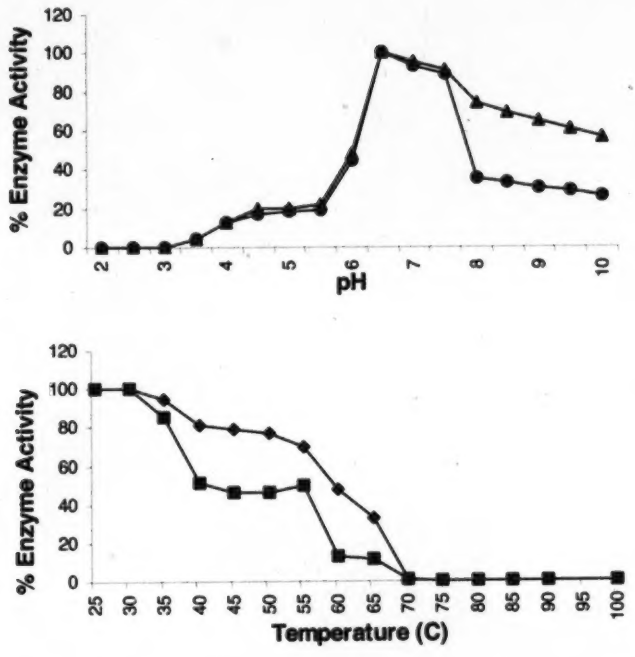

Figure 4.3. pH stability of GST at 4°C (▲) and room temperature (●) and temperature stability after heating for 1 min (◆) and 5 min (■).

4.3.3. *MALDI-TOF MS.*

Analysis of pure nisin by MALDI-TOF MS shows the presence of three peaks (Figure 4.4): a major nisin peak (MW = 3 359.9), nisin 1-32 (MW = 3 157.9) and a matrix adduct peak (MW = 3 572.0). The use of sinapinic acid as a matrix can result in the presence of two adduct peaks at increased masses of 207 and 224 Da; these two peaks tend to appear as one peak at ~215 Da (personal communication Randy Whitall). This was confirmed by MALDI-TOF MS analysis of nisin using HCCA, which is typically used as a matrix for low molecular weight peptides. The resulting spectra showed no

matrix adduct peaks; however, the resolution was poor compared with the spectra obtained with sinapinic acid as the matrix.

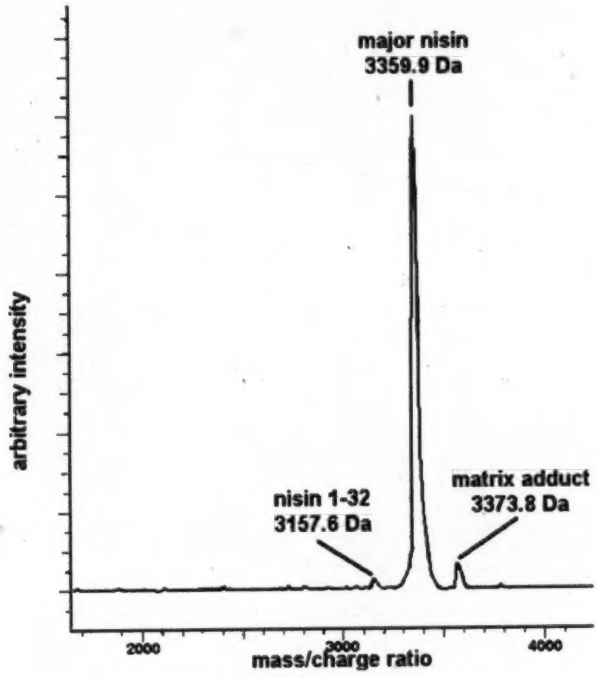

Figure 4.4. MALDI-TOF MS spectrum of pure nisin.

Analysis of the products of the reaction of nisin with glutathione showed a series of peaks with masses greater than that of nisin in increments of ~307 Da (Figure 4.5) at all reaction temperatures. The MALDI-TOF MS spectrum of the products of the reaction held at 4°C is similar to that seen previously in the meat assays (Rose et al., 1999; Chapter 3, this thesis); where it was hypothesized that the major peak indicated the addition of a single glutathione compound. As the reaction temperature was increased, it appeared that

multiple glutathione compounds reacted with nisin, which is indicative of multiple sites of reaction.

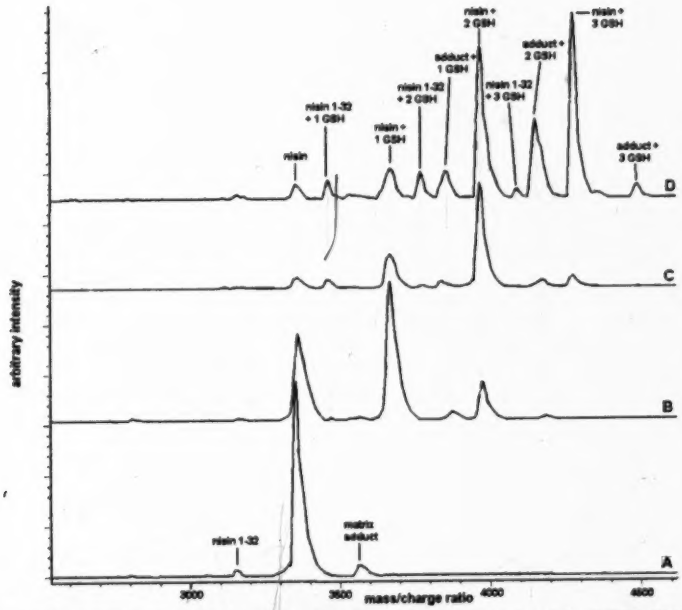

Figure 4.5. MALDI-TOF MS of (A) nisin (50 μg/mL) reacted with GSH (250 mM) in the presence of GST (1 mM) at (B) 4°C, (C) room temperature and (D) 37°C.

4.3.4. *Antibacterial activity assays.*

The products of the reaction of nisin and glutathione, with and without GST, were analyzed for antibacterial activity against *L. sakei* DSM 20017 and *C. divergens* NCFB 2855. The results are shown in Table 4.2. Complete loss of activity was observed against *C. divergens* NCFB 2855 under all conditions at 37°C and at room temperature. Loss of activity was observed in all cases against the more sensitive indicator, *L. sakei* DSM 20017. Complete loss of activity was not expected because the results of the MALDI-

TOF MS analysis (Figure 4.5) revealed the presence of unreacted nisin. Consistent with the characteristics of a GST substrate (Sipes and Gandolf, 1981), the reaction proceeds non-enzymatically at a slower rate.

Table 4.2. Antibacterial activity of nisin reacted with GSH, in the presence and absence of GST, at 37°C, room temperature and 4°C.

Temperature	37°				Room Temperature				4°C			
Indicator	*L. sakei* DSM 20017		*C. divergens* NCFB 2855		*L. sakei* DSM 20017		*C. divergens* NCFB 2855		*L. sakei* DSM 20017		*C. divergens* NCFB 2855	
Time	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
250 mM GSH + GST	51200	800	800	0	51200	1600	800	0	51200	6400	800	100
125 mM GSH + GST	51200	1600	800	0	51200	3200	800	0	51200	6400	800	200
50 mM GSH + GST	51200	1600	800	0	n.d.[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
250 mM GSH	51200	1600	800	0	51200	3200	800	0	51200	25600	800	200
125 mM GSH	51200	3200	800	0	51200	6400	800	0	51200	25600	800	400
50 mM GSH	51200	3200	800	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a]n.d. = not determined

4.3.5. *HPLC Chromatography.*

The HPLC profiles of nisin and the reaction products of nisin and glutathione are shown in Figure 4.6. Nisin was eluted at 23 min. The reaction products of nisin with glutathione are more water soluble than nisin; therefore, the products with multiple GSH reacted were eluted earlier, starting at 19 minutes. Different elution gradients were tried in an attempt to resolve the peaks; however, none were successful. Six fractions (pools A to F) from numerous HPLC runs were pooled and analyzed by MALDI-TOF MS and antibacterial activity assays.

4.3.6. *Analysis of HPLC fractions.*

Four HPLC fractions were pooled, dialyzed, freeze-dried and resuspended in phosphate buffer. Table 4.3 outlines the nisin-glutathione products that were present in each pooled sample. The samples were then assayed for antibacterial activity against a number of bacterial strains. The results are shown in Table 4.4. Pooled fractions where nisin was present were active against sensitive strains; otherwise, no activity was detected.

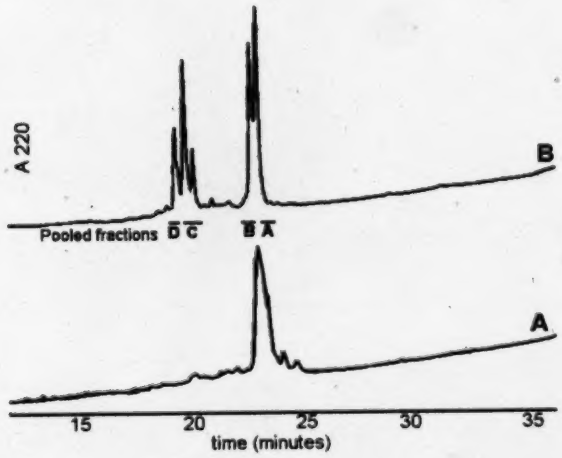

Figure 4.6. HPLC profile of (A) nisin and (B) nisin reacted with glutathione.

Table 4.3. Nisin-glutathione products present in HPLC fractions.

	Pool A	Pool B	Pool C	Pool D
Nisin	+	+	-	-
Nisin + 1 GSH	+	+	+	+
Nisin + 2 GSH	-	+	+	+
Nisin + 3 GSH	-	+	+	+
Nisin 1-32	+	+	-	-
Nisin 1-32 + 1 GSH	-	+	+	+
Nisin 1-32 + 2 GSH	-	-	+	+
Nisin 1-32 + 3 GSH	-	-	-	-

Table 4.4. Antibacterial activity of HPLC pooled fractions.

Indicator Organism	Pool A	Pool B	Pool C	Pool D
Listeria monocytogenes HPB 69	+	+	-	-
Listeria monocytogenes HPB 643	+	+	-	-
Lactobacillus sakei 706B	+	+	-	-
Lactobacillus sakei DSM 20017	+	+	-	-
Brochothrix campestris ATCC 43754	+	+	-	-
Pediococcus acidilactici PAC 1.0	+	+	-	-
Listeria monocytogenes Scott A	+	+	-	-
Listeria monocytogenes HPB 421	+	+	-	-
Lactobacillus sakei 706	+	+	-	-
Bacillus macerans ATCC 7048	+	-	-	-
Listeria monocytogenes List 4	+	-	-	-
Listeria monocytogenes ATCC 15313	+	-	-	-
Listeria monocytogenes HPB 65	+	-	-	-
Listeria monocytogenes HPB 66	+	-	-	-
Listeria monocytogenes HPB 67	+	-	-	-
Listeria monocytogenes HPB 68	+	-	-	-
Listeria monocytogenes HPB 70	+	-	-	-
Listeria monocytogenes HPB 121	+	-	-	-
Listeria innocua ATCC 33090	+	-	-	-
Listeria innocua CTC 1014	+	-	-	-
Lactobacillus curvatus 25601	+	-	-	-
Lactobacillus plantarum 4008	+	-	-	-
Leuconostoc gelidum UAL 187-13	+	-	-	-
Carnobacterium divergens UAL 278	+	-	-	-
Carnobacterium divergens NCFB 2855	+	-	-	-
Carnobacterium piscicola UAL LV17	+	-	-	-
Carnobacterium piscicola UAL 8C2	+	-	-	-
Carnobacterium piscicola UAL 26	+	-	-	-
Pediococcus acidilactici 8042	+	-	-	-
Staphylococcus aureus S13	+	-	-	-
Staphylococcus aureus 6538	+	-	-	-
Streptococcus bovis 15351	+	-	-	-
Enterococcus durans HPB 376	+	-	-	-
Enterococcus faecium ATCC 19434	+	-	-	-
Enterococcus faecium CTC 492	+	-	-	-
Enterococcus faecium BFE 900	+	-	-	-
Enterococcus faecalis ATCC 7080	-	-	-	-
Enterococcus faecalis ATCC 19433	-	-	-	-
Leuconostoc mesenteroides Y105	-	-	-	-
Lactococcus lactis ATCC 11454	-	-	-	-

4.4. Discussion.

Recent analysis of pure nisin in meat has led to the hypothesis that nisin reacts enzymatically with GSH and is subsequently inactivated (Rose et al., 1999; Chapter 3, this thesis). Here we have demonstrated that glutathione S-transferase is plentiful in fresh meat and that the reaction does proceed outside of a meat system and at various temperatures.

Among edible meat tissues, glutathione S-transferase has only previously been isolated from lean pork muscle (Williamson and Ball, 1988). The level of GST isolated from fresh beef is greater than the 0.06% reported in pig muscle (Williamson and Ball, 1988). The concentration in beef is closer to that of rat skeletal muscle in which GST constitutes about 0.1 to 0.3% of the total soluble protein (Ketterer, 1986). Similar to pork muscle tissue and unlike rat muscle tissue, it appears that beef muscle tissue contains a single form of glutathione S-transferase, as seen in two separate purifications. This is shown by the presence of a single band after both SDS-Page gel electrophoresis and isoelectric focusing. However, there is the possibility that multiple forms of GST with similar isoelectric points exist (Williamson and Ball, 1988). Of further interest is that the isolated enzyme retains most activity at a pH between 5.5 and 7.5 which is consistent with the pH of fresh beef ranging from 5.6 to 6.0.

Evidence supporting the reaction of nisin with mercaptans was provided by Liu and Hansen (1990). Their work convincingly showed that a reaction of nisin with either 2-mercaptoethane-sulfonate or thioglycolate resulted in a loss of antibacterial activity. In this experiment, a similar assay was performed with nisin and glutathione in the presence and absence of the glutathione S-transferase at 3 different temperatures. The objective of

this work was not to determine the enzyme kinetics of the reaction, but to confirm that the reaction proceeds and to analyze the reaction product(s).

An excess of GSH was used and the assays were held for 24 h in an attempt to have the reaction go to completion. However, minimal amounts of nisin remained unreacted as shown by MALDI-TOF MS (Figure 4.5) and the antibacterial activity assays (Table 4.2) against the less sensitive indicator strain, *L. sakei* DSM 20017. Whereas complete loss of activity was shown against *C. divergens* NCFB 2855 in assays performed at 37°C and room temperature. Consistent with known substrates of GST, the reaction also proceeds in the absence of the enzyme at a measurable rate (Sipes & Gandolf, 1991). In fact, in the presence of excess glutathione, the non-enzymatic reaction accounted for a major part of the reaction product (Parkenson, 1996). The apparent role of glutathione S-transferase in glutathione conjugation reactions is to increase the rate of reaction by assisting in the deprotonation of GSH to GS^- (Parkenson, 1996). The conjugation reaction can then proceed as a displacement reaction in which GS^- displaces an electron withdrawing group or as an addition reaction in which GS^- adds to an activated double bond. Addition reactions are facilitated by the presence of an electron-withdrawing group (e.g. –CN, -CHO, -COOR, -COR) attached to a double bond (Parkenson, 1996). Table 2 also shows that there is a loss of activity among control reactions. Previously, cooked meat assays with nisin did not show a loss of activity against *C. divergens* NCFB 2855 after storage at 4°C (Rose et al., 1999). This is likely explained by the slower reaction at refrigeration temperatures and the loss of glutathione after cooking. Wierzbicka et al. (1989) stated that the cooking of meats resulted in the conversion of GSH to forms that are not recoverable upon reduction.

Consistent with previous results (Rose et al., 1999; Chapter 3, this thesis), it was expected that a single glutathione molecule would react with nisin to yield loss of antibacterial activity. However, in the presence of an excess of glutathione and with an increase in reaction temperature, it was found that the addition of up to 3 glutathione molecules to one nisin molecule is possible (Figure 4.5). Under refrigeration conditions, the reaction proceeds at a slower rate and results primarily in the addition of one GSH molecule to nisin (Figure 4.5B). It is likely that the storage of meat at abusive temperatures could have resulted in multiple additions.

Given the nature of the dehydro residues, it was thought that the sites of addition could be the three dehydro residues at positions 2, 5 and 33. However, the fact that a peak corresponding to the addition of 3 glutathione molecules to nisin 1-32 exists (Figure 4.5D), it is likely that GSH adds elsewhere to the nisin molecule. Work is in progress to determine the primary site(s) of addition. Nisin 1-32 is a natural degradant of nisin and is expected to be present in purified nisin samples.

An attempt was made to separate the products of the reaction by HPLC. The addition of glutathione to nisin renders the antibacterial peptide more stable, and it is therefore eluted earlier from the HPLC column (Figure 4.6). Despite testing numerous conditions and elution profiles, we were unable to separate the compounds. However, analysis of the fractions by MALDI-TOF MS showed that nisin molecules with multiple glutathione molecules added were eluted earliest (Table 4.3). This is appropriate given the solubility of the compounds: the more glutathione added, the more soluble the compound. Antibacterial activity assays of the HPLC fractions confirmed the loss of activity of nisin upon reaction with GSH (Table 4.4).

This work has demonstrated that an active form of glutathione S-transferase is present in fresh beef. It has also provided conclusive evidence that the reaction between nisin and glutathione does occur. It has further demonstrated that this reaction not only occurs at one site, but at multiple sites on a nisin molecule.

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CHAPTER 5

INVOLVEMENT OF DEHYDROALANINE AND DEHYDROBUTYRINE IN THE ADDITION OF GLUTATHIONE TO NISIN

5.1. Introduction.

The lantibiotic nisin is an antibacterial peptide that is widely used as a natural preservative in the food industry. Lantibiotics, so-termed because of their characteristic sulfide ring formations, are small membrane-active peptides containing post-translationally modified amino acids (Klaenhammer, 1993). This involves the dehydration of serine and threonine to dehydroalanine and dehydrobutyrine, respectively. The dehydrated residues may then react with neighboring cysteine residues to form lanthionine or β-methyllanthionine residues.

Nisin exhibits antibacterial activity against a broad spectrum of gram-positive bacteria, including the spores of *Bacillus* and *Clostridium*. Nisin's activity is thought to be the result of two distinct killing mechanisms: 1) the N-terminal domain of nisin is able to interact with lipid II molecules and subsequently inhibit peptidoglycan synthesis, or 2) the C-terminal domain can translocate across the cell membrane to form pores and dissipate the proton motive force (Wiedemann et al., 2001). Furthermore, nisin activity against spores is likely through a reaction with sulfhydryl (-SH) groups of freshly germinated spores (Morris at al., 1984). The presence of the dehydrated amino acids and ring structures of nisin are essential for complete retention of antibacterial activity (Chan et al., 1996; Rollema et al., 1996; Giffard et al., 1997).

Nisin's high level of antibacterial activity, coupled with its heat and acid resistance, has resulted in its successful application in several dairy products, canned foods and vegetables (Delves-Broughton et al., 1996). However, nisin has had limited success in meat products. The use of nisin in meats is restricted to heat-treated meat products or its use in conjunction with other preservatives (Taylor et al., 1985; Fang and Lo Wei, 1994; Avery and Buncic, 1997; Davies et al., 1999; Barbuddhe et al., 1999; Ariyapitipun et al., 2000). The failure of nisin to inhibit target bacteria in meat products was hypothesized to be the result of binding to meat components and surfaces, sensitivity to food enzymes, interactions with phospholipids, poor solubility of nisin, or uneven distribution of nisin on the meat product (Scott and Taylor, 1981; Bell and de Lacy, 1986; Stringer et al., 1995; Henning et al., 1986; Cutter and Siragusa, 1998).

Our laboratory hypothesized that nisin was inactivated in fresh meat by an enzymatic reaction with glutathione, a low molecular weight (307 Da) thiol compound found in meat tissues (Rose et al., 1999; Chapter 3, this thesis). The *in vitro* reaction between pure nisin and glutathione confirmed that, under optimum conditions, glutathione adds to multiple sites on a nisin molecule (Rose et al., 2001; Chapter 4, this thesis). The theory is that the dehydro residues of nisin are able to act as Michael acceptors towards sulfhydryl groups (Liu and Hansen, 1990). The purpose of this study was to determine the sites of addition of the glutathione molecules to better understand the loss of activity of nisin in meat products. Nisin variants were reacted with glutathione, and the products of the reactions were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography coupled with mass spectrometry techniques (LC-MS). The chemical

structure of the products of the nisin and glutathione reaction were also compared with the native nisin molecule using nuclear magnetic resonance (NMR).

5.2. Materials and methods.

5.2.1. *Construction of nisin variants.*

Nisin variants and fragments were constructed and purified at the Institute of Food Research (Norwich, UK). Protein-engineered [Ala5], [Ala33] and [Ala5+33] nisin variants were produced and purified by means of an expression system as described by Dodd et al. (1996). Briefly, a lactococcal expression system was developed using a gene replacement technique in which variant *nis*A genes were substituted for the chromosomal wild-type gene. Fragments of nisin were generated enzymatically by proteolytic digestion with α-chymotrypsin ($Nisin_{1-20}$) or trypsin ($Nisin_{1-12}$) and purified as described by Chan et al. (1996). The structure of nisin highlighting the variants and fragments is shown in Figure 5.1. The relative molecular masses of the nisin A variants and fragments were confirmed by MALDI-TOF MS as described below.

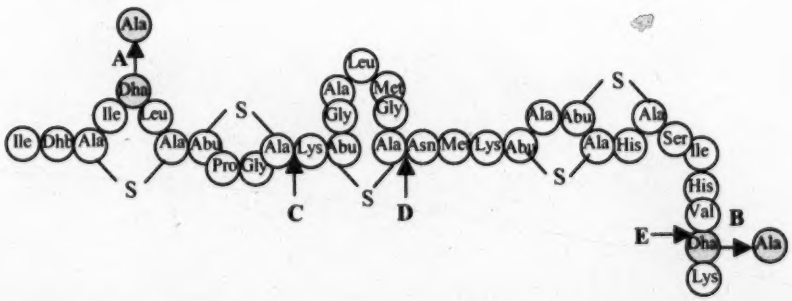

Figure 5.1. Structure of nisin, indicating the sites of variation and fragmentation: (A) [Ala5] nisin A; (B) [Ala33] nisin A; (AB) [Ala5+Ala33] nisin A; (C) nisin 1-12; (D) nisin 1-20, and (E) nisin 1-32.

5.2.2. *Nisin assay.*

Glutathione S-transferase was purified from fresh beef, as previously described by Williamson and Ball (1988). Stock solutions of nisin and the nisin variants (100 μg/mL) and purified glutathione S-transferase (1 mM) were dissolved in 50 mM sodium phosphate buffer (pH 6.0). Glutathione was dissolved in the sample buffer at a concentration of 50 mM, and the solution was pH adjusted to 6.0. Final working assays consisted of glutathione (25 mM), nisin (50 μg/mL) and glutathione S-transferase (1 μM). Assays were carried out at the optimum temperature of 37°C. The reactions were stopped after 24 h by adding TFA to a final concentration of 0.3%. The products were analyzed by MALDI-TOF MS and LC-MS.

5.2.3. *MALDI-TOF MS analysis.*

All mass spectra data were acquired on a linear MALDI-TOF MS equipped with pulsed ion extraction technology (Bruker Proflex™ III, Billerica, MA) and a 125 cm flight tube, in a positive ion linear mode with a nitrogen laser (λ =337 nm) for desorption/ionization of the samples and an acceleration voltage of 20 kV. Samples were prepared for MALDI-TOF analysis using the dried droplet method (Kussmann et al., 1997). A saturated solution of the sinapinic acid matrix (Sigma Chemical Co., St. Louis, MO) was prepared in a solution containing two parts of 0.1% TFA and one part of acetonitrile. The sample was mixed with the matrix (1:1), spotted on a stainless steel probe, and allowed to air dry.

5.2.4. *LC-MS analysis.*

All nisin variants and their fragments and reaction products were analyzed using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, UK), coupled to a Hewlett-Packard 1050 quaternary pump HPLC system equipped with 1050 autoinjector and 1050 Diode Array detector (Agilent Technologies UK Ltd., Stockport, UK). Spectra were obtained in positive ion electrospray mode using a Micromass Z-spray™ ion source. The electrospray probe was operated at 3.5 kV and the cone voltage was 28 V. The source and desolvation temperatures were 120° and 350°C respectively. The nitrogen nebulising and drying gas flow rate were optimised at 15 L/h and 350 L/h, respectively. Spectra were recorded scanned in continuum (i.e., raw data acquisition) by cyclic scanning of the mass range m/z 400 to 2200, with a scan duration of 5.0 s and an interscan time of 0.2 s. The mass spectrometer was set to unit mass resolution or better (LM and HM resolution parameters both at 15.0).

HPLC chromatographic conditions were as follows: solvent A: 0.1% trifluoroacetic acid (TFA) in Milli-Q purified water, Solvent B: 0.1% TFA in HPLC grade acetonitrile; flow rate 1 mL/min; gradient 0.0 to 100% B in 45 min. A Phenomenex® Jupiter C_4 column (250 × 4.60 mm packed with 5 µm beads; Torrence, CA) was used. The flow was split between the mass spectrometer and diode array detector in the approximate ratio of 1:8. Diode array spectra were recorded simultaneously with mass spectra in the range 190 to 600 nm.

5.3. Results.

5.3.1. *MALDI-TOF MS and LC-MS analyses.*

MALDI-TOF MS and LC-MS analyses were performed on the nisin variants and fragments prior to the reaction with glutathione to confirm their molecular weight and to determine whether any degradation fragments were present in the sample. The experimental masses are shown in Table 5.1. There is a discrepancy in the molecular masses as reported by MALDI-TOF MS and LC-MS. For example, the masses of the single variant nisin, as reported by MALDI-TOF MS, were lower than nisin, whereas with LC-MS, they were higher. This is likely due to difficulty in determining the peak centroid due to the low resolving capabilities of MALDI-TOF MS (Wang et al., 1998).

Table 5.1. Molecular masses of nisin variants and fragments determined by MALDI-TOF MS and LC-MS.

Nisin fragment/variant	Experimental mass (MALDI-TOF MS)	Experimental mass (LC-MS)
Nisin	3362.0	3353.6
[Ala5] nisin	3360.3	3355.6
[Ala33] nisin	3359.3	3355.6
[Ala5+33] nisin	3366.3	n.d.
Nisin 1-32	3184.5	3185.6
Nisin 1-20	1880.7	n.d.
Nisin 1-12	1150.6	1150.1

n.d. not determined

MALDI-TOF MS was also used to examine the products of the reaction between the nisin variants and fragments and glutathione. A shift in mass of increments of ~+307 Da was used to determine the number of glutathione molecules that were added to the

nisin molecules. Figures 5.2 and 5.3 show the mass spectra of the products of the reactions.

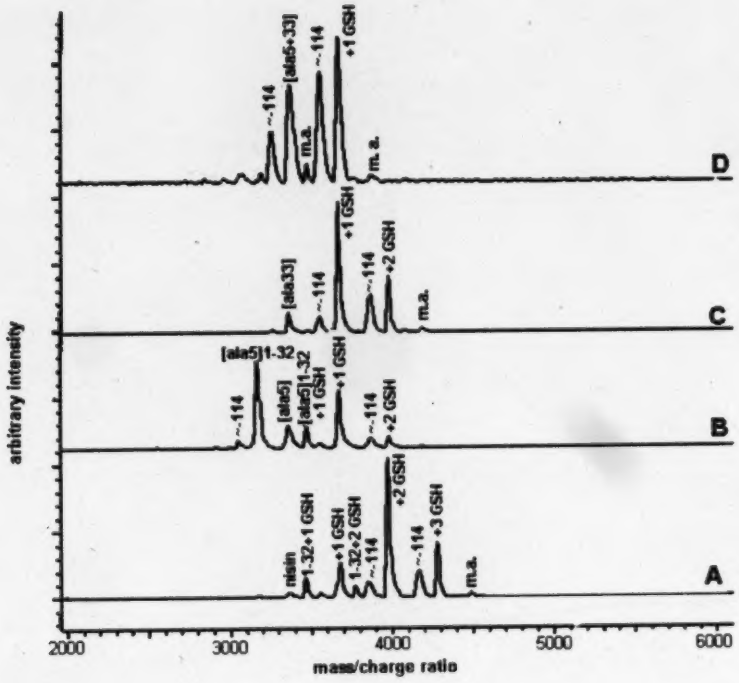

Figure 5.2. MALDI-TOF MS spectra of the products of the reaction between glutathione and (A) nisin; (B) [Ala5] nisin; (C) [Ala33] nisin, and (D) [Ala5+33] nisin; m.a. refers to the presence of a matrix adduct.

A nisin molecule has 3 dehydro residues that are not linked to a cysteine residue by a thioether bond (Dhb2, Dha5 and Dha33); whereas [Ala5] nisin and [Ala33] nisin have 2 dehydro residues (Dhb2 and Dha33, and Dhb2 and Dha5, respectively) and [Ala5+33] nisin has 1 dehydro residue (Dhb2). The products of the reaction between nisin and glutathione showed that up to 3 glutathione molecules can be added to one nisin molecule (Figure 5.2A). In regards to the products of the reaction between glutathione

and the nisin variants, it shows that two glutathione molecules can be added to the single variants (Figure 5.2B and 5.2C) and that one glutathione molecule can be conjugated to the double variant of nisin (Figure 5.2D). These data also show an instability of the [Ala5] nisin molecule (Figure 5.2B). It appears upon reaction of nisin with glutathione, the two C-terminal amino acids are cleaved; whereas this is not apparent before the reaction with glutathione (Table 5.1). After cleavage, [ala5] $nisin_{1-32}$ has only 1 dehydro residue (Dhb2), and undergoes the addition of a single glutathione molecule. The MALDI-TOF MS data showed that the number of glutathione molecules that can add to either nisin or the nisin variants is consistent with the number of dehydro residues in the molecule. The MALDI-TOF MS analysis also showed the presence of matrix adduct peaks of ~+215 Da, which is typical of the use of sinapinic acid as the matrix (personal communication, Randy Whittal).

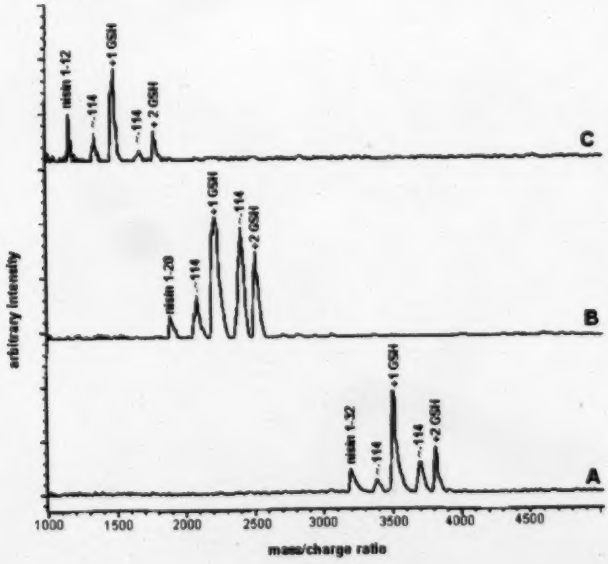

Figure 5.3. MALDI-TOF MS spectra of the products of the reaction between glutathione and (A) nisin 1-32; (B) nisin 1-20, and (C) nisin 1-12.

Fragmentation of the nisin molecule involved cleavage of the C-terminus region at the positions after the 12, 20 and 32 amino acid residues. Each of the fragments retained 2 dehydro residues (Dhb2 and Dha5). The MALDI-TOF MS data, in Figure 5.3, shows that two glutathione molecules were added to each of the fragments, even when only the first 12 residues of nisin were studied. This suggests that the N-terminus of the nisin molecule is highly susceptible to nucleophilic attack by glutathione, and that the site of the third glutathione addition is in the C-terminus region.

In addition to the peaks corresponding to the addition of the glutathione molecule(s), a number of other fragmentation peaks was evident from the MALDI-TOF MS and the LC-MS data. Table 5.2 lists the dominant peaks. The dominant fragment peaks are consistent loss of either ~114 Da, as detected by MALDI-TOF MS and LC-MS, or ~129 Da, as detected by LC-MS. Screening of the reaction products by a reflectron MALDI-TOF mass spectrometer also showed the fragment peaks of less than ~129 Da (data not shown). Because the peaks are seen in each of the product samples, it is likely that the fragmentation occurs at the N-terminal region of the molecule. This could be the result of a common phenomenon with mass spectrometry techniques, called post-source decay, which results in the fragmentation of the analyte molecule (Jiménez and Burlingame, 1998). This phenomenon is not typically seen with linear MALDI-TOF mass spectrometry, but may be seen with other mass spectrometry instrumentation. Since the loss of ~114 Da was seen with the linear mass spectrometer (Figures 5.2 and 5.3) and since the loss of ~114 Da was seen with the parent molecule of [Ala5+33] nisin (Figure 5.3D) after the reaction with glutathione and not prior to the reaction (data not shown), it is likely that this cleavage occurs in nature. To determine the sites of cleavage, the

monoisotopic mass of nisin after the addition of two GSH molecules was compared with the monoisotopic masses after cleavage at two different sites as shown in Figure 5.4.

Table 5.2. Summary of the dominant peaks of the products of the reaction between the nisin variants and fragments with GSH after analysis by MALDI-TOF MS and LC-MS.

	MALDI-TOF MS		LC-MS	
	Experimental mass	Mass difference	Experimental mass	Mass difference
Nisin	3362.0		3353.6	
Addition of 1 GSH molecule	3669.5	+307.5		
Addition of 2 GSH molecules	3976.2	+614.2	3968.0	+614.4
Fragment peaks	3860.2	-116.0	3839.0	-129
Addition of 3 GSH molecules	4283.3	+921.3	4275.2	+921.6
Fragment peaks	4169.0	-114.3	4161.3	-113.9
			4146.2	-129.0
[Ala5] nisin	3360.3		3355.6	
Addition of 1 GSH molecule	3667.8	+307.5	3662.9	+307.3
Fragment peaks	3554.6	-113.2		
Addition of 2 GSH molecules	3976.0	+615.7	3970.2	+614.6
Fragment peaks	3862.1	-113.9	3856.1	-114.1
			3840.7	-129.5
[Ala33] nisin	3359.3		3355.6	
Addition of 1 GSH molecule	3666.5	+307.2	3662.7	+307.1
Fragment peaks	3552.4	-114.1		
Addition of 2 GSH molecules	3973.1	+613.8	3969.7	+614.1
Fragment peaks	3859.1	-114.0	3855.9	-113.8
			3840.6	-129.1
[Ala5+33] nisin	3366.3			
Addition of 1 GSH molecule	3673.5	+307.2	n.d.	
Fragment peaks	3560.4	-113.1		
Nisin 1-32	3184.5		3185.6	
Addition of 1 GSH molecule	3492.0	+307.5	2878.5	+307.1
Fragment peaks	3377.0	-115.0		
Addition of 2 GSH molecules	3799.0	+614.5	3800.2	+614.6
Fragment peaks	-3687.1	-111.9	3685.9	-114.3
			3671.0	-129.2
Nisin 1-20	1880.7			
Addition of 1 GSH molecule	2187.6	+306.9		
Fragment peaks	2073.3	-114.3	n.d.	
Addition of 2 GSH molecules	2494.0	+306.4		
Fragment peaks	2382.7	-111.3		
Nisin 1-12	1150.6		1150.1	
Addition of 1 GSH molecule	1457.7	+307.1		+307.3
Fragment peaks	1343.6	-114.1		
Addition of 2 GSH molecules	1763.8	+613.2	1764.7	+614.6
Fragment peaks	1648.1	-115.7	1650.5	-114.2
			1635.5	-129.2

n.d. not determined

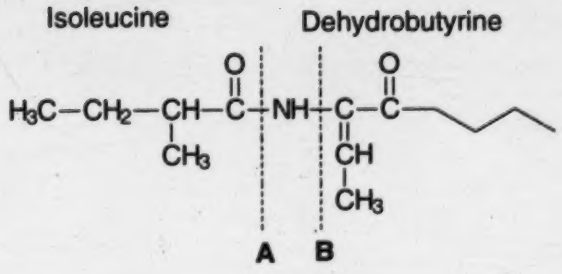

Figure 5.4. The structure of the first two amino acids in the N-terminus region of nisin, indicating the potential sites of cleavage.

The monoisotopic mass of nisin with two GSH molecules added ($C_{163}H_{265}N_{48}O_{49}S_9$), as determined by MS-Isotope 1.3.1, Protein Prospector 3.4.1 (The Regents of the University of California), is 3966.7206. Cleavage at the peptide bond between isoleucine and dehydrobutyrine results in a monoisotopic mass of 3852.6287 ($C_{157}H_{253}N_{47}O_{48}S_9$) and cleavage after the amine group results in a monoisotopic mass of 3837.6179 ($C_{157}H_{252}N_{46}O_{48}S_9$). The mass differences compared with the major nisin molecule with two GSH added are 114.0919 and 129.1027, respectively.

5.3. Discussion.

The nisin molecule is a complex antibacterial peptide having 13 of 34 residues that are post-translationally modified to dehydro residues and thio-ether cross-linkages. Several studies have demonstrated the importance of such modifications to the antibacterial efficacy of nisin. In particular, the acid-catalyzed addition of a water molecule to Dha5 results in a loss of integrity of ring A of nisin, and subsequently, a decrease in its antibacterial activity (Rollema et al., 1996). Similarly, cleavage of ring C

in nisin results in the complete loss of activity (Chan et al., 1996). However, the dehydro residues are not essential for nisin activity. The transformation of [Ala5] nisin, [Ala33] nisin or [Ala5+33] nisin variants retained some activity (Dodd et al., 1995). However, [Ala5] nisin was much less active against the outgrowth of spores of *Bacillus subtilis* (Chan et al., 1996), supporting the evidence that the dehydroalanine residues are involved in the killing action against bacterial spores (Morris et al., 1984).

The inhibition of nisin by glutathione was suggested to be similar to that of the mode of action of nisin against spores (Rose et al., 1999; Chapter 3, this thesis). It is assumed that nisin interacts with a sulfhydryl group on the outer membrane of freshly germinated spores (Morris et al., 1984). Nisin is a suitable agent for such a reaction because its dehydro residues can act as potential Michael acceptors to react with the sulfhydryl groups. The addition reaction between glutathione and nisin becomes even more likely to occur with the presence of an electron withdrawing group (-COR) next to the conjugated double bond system. This and the fact that up to three glutathione molecules could react with one molecule of nisin led to the working hypothesis that Dhb2, Dha5 and/or Dha 33 could react with nisin and result in its loss of activity, as previously reported in Chapter 4 of this thesis (Rose et al.,2001). The involvement of the dehydroalanine residues in the reaction between nisin and glutathione was clearly demonstrated by the assays with the nisin variants and fragments. It is hypothesized that the dehydrobutyrine residue is the third site of addition for glutathione to nisin. This is made on the basis that 2 glutathione molecules can add to one $nisin_{1-12}$ fragment, which contains 2 dehydro residues. Addition of glutathione across the carbon-carbon double

bond of the dehydro residues, as shown in Figure 5.5, results in the loss of antibacterial activity of nisin.

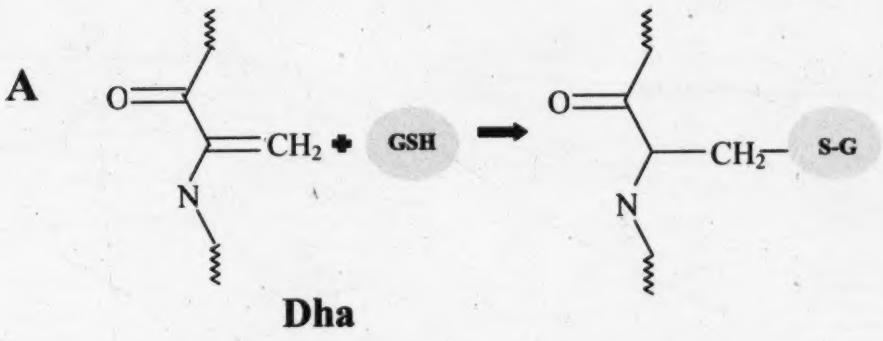

Figure 5.5. Proposed reaction of addition of glutathione to dehydroalanine.

There was no specificity for either the Dha5 or Dha33 amino acid residues because the reaction proceeded with all nisin variants.

This work has provided an insight into the potential use of nisin in meat products. It appears that the addition of glutathione to nisin is one reason why nisin has been ineffective as a natural preservative in fresh meats. However, the successful application of nisin to meats may be achieved with: 1) the addition of nisin to heat-treated products, as demonstrated by Davies et al. (1999); 2) the use of an active form of nisin without available dehyro residues, or 3) the use of a food-grade glutathione S-transferase inhibitor in fresh meat products.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The lactic acid bacteria have been isolated from a variety of foods, and they are commonly used as defined starter cultures in fermented foods. The presence of a lactic acid bacteria microflora in foods is typically advantageous because of the production of fermentation end products that can result in the inhibition of other microorganisms. Several strains of lactic acid bacteria provide an additional protective effect to foods through the production of antibacterial peptides called bacteriocins. Bacteriocins inhibit strains of bacteria that are generally closely related to the producer strain (Klaenhammer, 1993).

Consumer trends are demanding fresh and natural foods, which means that the food industry is looking to minimize the use of synthetic chemical additives as food preservatives. Therefore, the opportunity for the application of bacteriocins to foods has become attractive as a means of natural preservation. The bacteriocins of lactic acid bacteria could be added to food via the producer organism or as a partially-purified commercial preparation or in a purified form. To date, one bacteriocin is licensed for use in foods. Nisin is approved for use in dairy products, canned foods and vegetables in over 50 countries worldwide (Delves-Broughton et al., 1996). Despite such success, nisin is not used in meat products because of the interference of meat components (Henning et al., 1986). Clarification of the fate of nisin in meats is needed to enhance our understanding of the role that nisin could play in the preservation and safety of meat

products. Obtaining such an understanding has been hampered by our inability to detect bacteriocins in complex food matrices at low concentrations. Therefore, there was a need to develop sensitive methods for the detection of bacteriocins, and perhaps their degradation products, in complex matrices such as meat.

In recent years, the emergence of MALDI-TOF MS for the detection of large biomolecules has rapidly advanced the analysis of peptides and proteins in crude biological samples. This method involves co-crystallizing the sample with a suitable matrix; the matrix strongly absorbs the energy from pulsed laser beams and subsequently fragments and forms analyte ions that are ejected into a vacuum for desorption analysis. MALDI-TOF MS is advantageous over other forms of mass spectrometry because of its ease of use, picomole-to-femtomole sensitivity, high mass range, limited ion fragmentation, and relative tolerance to contaminants in the sample (Siuzdak, 1994; Blackledge and Alexander, 1995a,b; Costello, 1997; Whittal and Liang, 1997; Wang et al., 1998; Worrall et al., 1998). Although MALDI-TOF MS is tolerant to many contaminants, analysis of samples from biological sources has been hampered by the presence of salts, glycerol and detergents (Blackledge and Alexander, 1995a,b; Kussmann et al., 1997; Amado et al., 1997; Worrall et al., 1998). These contaminants may result in complete ion suppression or the preferential detection of certain peptides over others. This is a major concern when considering the use of MALDI-TOF MS for the detection of bacteriocins.

Common growth media for bacteriocin-producing LAB include APT and Lactobacillus MRS that contain the detergent Tween 80, which is an essential growth component for maximum bacteriocin production (Joosten and Nũnez, 1995; Huot et al.,

1996). It was likely that the presence of Tween 80 in the bacteriocin sample would interfere with MALDI-TOF MS analysis. Therefore, it would be necessary to either purify the sample prior to analysis or to consider an on-probe clean-up method. Activated membranes, on-probe immunoaffinity purification and on-probe washes have been used to minimize the interference of sample contaminants (Blackledge and Alexander, 1995a, b; Worrall et al., 1998; Liang et al., 1998). Each method resulted in greater sensitivity and mass resolution for all samples.

As expected, the presence of Tween 80 in the culture growth medium completely suppressed the ion signal of bacteriocins. However, an on-probe water wash effectively washed away the contaminants, and MALDI-TOF MS detected bacteriocins in the supernatant of the growth medium (Rose et al., 1999a; Chapter 2, this thesis). This involved allowing the sample to dry on the target, washing the sample with water, and then applying the matrix on top of the sample. To our knowledge, this was the first reported mass spectrometry method for the detection of bacteriocins from culture supernatant. This method was also used to identify bacteriocins in various fractions throughout the bacteriocin purification process. This eliminated the need to wait 12 to 24 hours for results from activity assays. In comparison, MALDI-TOF MS analysis takes minimal time (i.e., minutes). The ability of MALDI-TOF MS to detect bacteriocins from a complex medium is largely due to its ability to tolerate a mixture of compounds in a sample and the ability to partially purify samples on the sample probe. This makes it attractive for use in the detection of bacteriocins in genetic and food experiments.

As mentioned above, nisin has been ineffective as an antibacterial peptide in meat systems. To date, there was no scientific evidence providing a possible mechanism of the

inactivation of nisin in meat. Therefore, it was hypothesized that MALDI-TOF MS may provide some insight into the fate of nisin in meat. This could be possible by the detection of either degradation peaks of the nisin molecule or peaks showing an increase in mass from the nisin molecule, suggesting either an enzymatic degradation of nisin or the binding of nisin to meat components, respectively.

To examine the potential of MALDI-TOF MS for the detection of bacteriocins in foods, purified nisin was added to meat and stored overnight. MALDI-TOF MS and antibacterial activity assays detected the presence of nisin from an extract of a cooked meat sample after 24 h of storage. However, the same was not true when attempts were made to recover nisin from either fresh beef or pork. MALDI-TOF MS analysis of extracts of fresh meat that had been treated with nisin showed the presence of a compound ~307 Da greater than the expected mass of nisin, and no antibacterial activity was recovered. All evidence led to the hypothesis that nisin had been inactivated through a reaction with glutathione. This hypothesis was further supported by a statement made by Gross and Morell (1967) in their study of the relationship of dehydroalanine to nisin activity: "Metabolically important compounds, such as sulfhydryl-containing enzymes, glutathione, or coenzyme A, may be intercepted by nisin."

Glutathione is a tripeptide consisting of glutamic acid, cysteine and glycine, with the most reactive component being the thiol group of the cysteine residue. Glutathione is a major low molecular thiol found in plants and animals, and it appears to be limited to aerobic organisms. Because glutathione is a component of plant and animal tissues, it is found in a variety of foods. Wierzbicka et al. (1989) showed that the glutathione content varies in fruits and vegetables (21 to 552 nmol/g) and meats (156 to 630 nmol/g), with

lesser amounts found in fish (21 nmol/g). In contrast, milk, eggs and butter have essentially no glutathione. Interestingly, nisin is widely used as an effective antibacterial in dairy products.

Reactions that involve glutathione are typically enzyme-catalyzed. This could explain why there was no loss of nisin activity in the cooked meat sample; the reaction did not occur because of the inactivation of the enzyme after heating. Lee et al. (1996) reported that glutathione S-peroxidase was present in high amounts in fresh turkey meat; however, after cooking, minimal amounts of the enzyme were recovered. It is known that reactions with glutathione can proceed without the enzyme; however, they proceed at a slower rate than the enzyme-catalyzed reaction. However, with an excess of glutathione, the non-enzymatic reaction can account for a major proportion of the reaction product(s) (Parkenson, 1996). In the current studies with meat, the non-enzymatic reaction was not likely to occur because of the low storage temperatures. Furthermore, there is the possibility of the loss of glutathione during cooking, which could account for lack of non-enzymatic reaction. Lee et al. (1996) showed that the amount of glutathione in poultry meat was unaffected by heat. In contrast, Wierzbicka et al. (1989) reported that cooking of meat could result in the conversion of glutathione to forms that are not recoverable upon reduction.

The enzyme responsible for the reaction between glutathione and nisin was confirmed to be glutathione S-transferase. Glutathione S-transferase was isolated from fresh beef and used to catalyze the reaction (Rose et al., 1999b; Chapter 4; this thesis). Glutathione S-transferase catalyzes the addition of glutathione to compounds containing electrophilic carbon atoms, forming a thio-ether bond between the carbon atom and the

sulfhydryl group of the cysteine residue in glutathione (Sipes and Gandolf, 1991). Nisin contains many features common to known substrates of glutathione S-transferase, including its hydrophobic nature and the presence of electrophilic carbon atoms in the dehydro residues (Sipes and Gandolf, 1991).

Glutathione S-transferases comprise a family of dimeric enzymes that catalyze the conjugation of glutathione to a wide spectrum of compounds that contain electrophilic groups (Habig and Jakoby, 1981). As with glutathione, these enzymes are typically found in most aerobic microorganisms, plants and animals. Conjugation reactions catalyzed by glutathione S-transferase are of two types: (1) a displacement reaction in which glutathione displace and electron withdrawing group or (2) an addition reaction in which glutathione is added to an activated double bond or strained ring system (Parkenson, 1996). The latter reaction is facilitated by the presence of a nearby electron-withdrawing group.

Substrates for glutathione conjugation share three common features: they are (1) hydrophobic to some degree; (2) contain an electrophilic atom, and (3) react nonenzymatically with glutathione to some degree (Sipes and Gandolf, 1991). Therefore, nisin is a prime candidate for such a sulfhydryl agent because it is a hydrophobic peptide with unusual dehydro residues that could act as electrophilic Michael acceptors and readily react with mercaptans and other nucleophiles (Morris et al., 1984; Liu and Hansen, 1990). The dehydro residues are also the most likely site because of their system of conjugated double bonds and the presence of the neighboring –COR group. Furthermore, dehydroalanine has been shown to target membrane sulfhydryl groups in

newly germinated spores to inhibit the outgrowth of *Bacillus cereus* and *Clostridium botulinum* spores (Somers and Taylor, 1981; Scott and Taylor, 1981; Morris et al., 1984).

To confirm the *in vitro* reaction between nisin and glutathione and to further study the site of the reaction, glutathione S-transferase was purified from fresh meat by a series of chromatography methods and was used to study the reaction of nisin with glutathione. A single form of the enzyme was isolated showing the appropriate range for subunit size and isoelectric point (Habig and Jakoby, 1981). The enzyme retains most of its activity in the pH range of 5.5 and 7.5. With an excess of glutathione, the reaction between nisin and glutathione readily proceeded with little difference in the remaining antibacterial activity of nisin between the nonenzymatic and enzymatic reactions. Based on the results of reactions of glutathione and nisin in meat, it was expected that only one glutathione would conjugate to a single nisin molecule as seen in the meat study (Rose et al., 1999b; Chapter 3, this thesis). As the temperature of the reaction increased to the optimum temperature of 37°C, the reaction products consisted of up to 3 glutathione molecules conjugating to one nisin molecule.

The focus of the research then turned to determining the site of the reaction of glutathione and nisin. Consistent with known substrates of glutathione as described above, it was assumed that the three dehydro residues of nisin were the likely targets of glutathione conjugation. It is known that the dehydro residues are not essential for activity against vegetative cells (Dodd et al., 1996); therefore if the dehydro residues were confirmed to be the sites of addition, there would be the possibility of using nisin variants lacking the dehydro residues as antibacterial agents in meat products. Confirmation of the

involvement of the dehydro residues was done using variants and fragments of the nisin molecule obtained from genetic engineering.

Several variants of nisin have been generated by researchers at the Institute of Food Research (Norwich, UK). The ones of interest in this study had modified dehydro residues, including an alanine at Dha5, and alanine at Dha33 and a double variant with alanines at both the Dha5 and Dha33 positions (Dodd et al., 1995, 1996). This resulted in nisin variants with either one or two dehydro residue(s) instead of the natural three residues in nisin. As expected, the results of various mass spectrometry techniques showed that the maximum number of glutathione molecules conjugated to a nisin molecule was consistent with the number of dehydro residues in nisin or the nisin variants. $Nisin_{1-32}$, $nisin_{1-20}$ and $nisin_{1-12}$ fragments were also used in reactions with glutathione. Each fragment had a maximum of two glutathione molecules added, which demonstrated that two of the possible three glutathione molecules are conjugating to the N-terminus region of the nisin molecule. These experiments confirmed the involvement of Dha5 and Dha33 in the reaction; however, it failed to provide conclusive evidence of the involvement of Dhb2. This work provided the first scientific evidence for the inactivation of nisin in fresh meat systems. It also supported previous evidence that disruption of the dehydro residues resulted in the inhibition of nisin.

The research fulfilled the objectives of this thesis as outlined previously. The primary objectives involved establishing MALDI-TOF MS as a means to detect bacteriocins. Detection of several bacteriocins from the culture supernatant was achieved with a simple on-probe wash with water. This technique was also used to track a

bacteriocin throughout a purification procedure. This established the grounds for the application of MALDI-TOF MS in the study of bacteriocins in complex matrices such as meat. Nisin was able to be detected from an extract of cooked meat; however, MALDI-TOF MS analysis and antibacterial activity assays of a fresh meat extract suggested that nisin was inactivated in fresh meat by an enzymatic reaction with glutathione. These results sparked further interest in this phenomenon and a number of secondary objectives were proposed. These were met by the isolation of glutathione S-transferase from fresh beef and studies of the *in vitro* reaction between nisin and glutathione. All results confirmed that glutathione reacted with nisin at any one of the three available dehydro residues. This work suggests that the potential use of nisin in meat products is limited to: (1) the use of nisin in heat-treated meat products; (2) the use of an active form of nisin without dehydro residues, or (3) the use of nisin along with a food-grade glutathione-S transferase inhibitor in fresh meat.

The first option has already been shown to be effective by Davies et al. (1999). The potential for the use of an active form of nisin without dehydro residues seems to be limited. Studies have shown that the although [Ala5] nisin, [Ala33] nisin and [Ala5+33] nisin are active, changing Dha5 to an alanine residue results in a 50% loss of antibacterial activity as compared to the wild-type nisin (Dodd et al., 1995). The effects on the antibacterial activity of nisin with all dehydro residues altered is not yet known and must be determined before application in meat products. The potential of using a food-grade glutathione S-transferase inhibitor is also a complex solution. The inhibitor must work in the system to first allow the effective use of the antibacterial nisin and secondly to not change the properties of the meat products.

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